Characterisation of the virus-specific $CD8^+$ T cell response in acute and early HIV-1 infection

By

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Abstract

The primary human immunodeficiency virus type 1 (HIV-1) - specific $CD8^+$ T cell response plays an important role in control of early viral replication. The nature of this response may thus be among the factors determining the prognostically-important persisting viral load. Here, the primary HIV-specific $CD8^+$ T cell response was characterised in a small cohort of patients, to gain insight into quantitative and qualitative features of the response and their relationship to the efficiency of control of virus replication.

The majority of patients studied established intermediate or high persisting viral loads. In these individuals, no association was found between the persisting viral load established and the total magnitude of the early HIV-specific $CD8^+$ T cell response, its epitope breadth or specificity. However the response was observed to be heavily biased towards the most immunodominant epitopes.

Kinetic analyses of epitope-specific responses revealed that in most patients, responses expanded asynchronously, with rapidly-expanded responses being immunodominant in primary infection. Mechanisms that may account for the rapid initial expansion of selected responses in acute viral infection, including high avidity and pre-existence of cross-reactive memory cells, were explored using murine models.

Notably, the most rapidly-expanded epitope-specific response(s) typically did not reach peak magnitude until several weeks after the peak in acute viral replication; and the fastest response was observed in the one patient studied who established a low persisting viral load.

Phenotypic studies suggested that HIV-specific $CD8^+$ T cells exhibit an "immature" phenotype from primary infection onwards in all patients.

Although these studies need to be extended to a larger number of patients, the results obtained suggest that the kinetics and synchronicity of expansion of epitope-specific $CD8^+$ T cell responses in primary HIV-1 infection may be among the factors that impact on the efficiency of control of primary viraemia.

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List of abbreviations used

2' 5' OAS	2' 5' oligoadenylate synthetase
aa	Amino acid
$\alpha\beta$	Alpha beta
ADCC	Antibody-dependent cell-mediated cytotoxicity
AICD	Activation-induced cell death
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
AP	Alkaline phosphatase
APC	Antigen presenting cell
APOBEC (3G/3F)	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like (3G/3F)
ART	Antiretroviral therapy
ASK-1	Apoptosis signalling regulating kinase- 1
AZT	Azidothymidine
β_2m	β_2 microglobulin
BCG	Bacillus Calmette-Guérin
β gal	β galactosidase
BIMAS	BioInformatics and Molecular Analysis Section
BPV	Bovine papilloma virus
BrDU	5'-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CAF	$CD8^+$ cell-derived anti-HIV-1 inhibitory factor
CD	Cluster of differentiation
cDNA	Complementary DNA
CMV	Cytomegalovirus
CNS	Central nervous system
CO_2	Carbon dioxide
Con A	Concanavalin A
CR	Complement receptor
CRF	Circulating recombinant form
Crm1	Chromosomal region maintenance 1
CTD	Carboxyl terminal domain
CTL	Cytotoxic T lymphocyte
DAF	Decay-accelerating factor
DC	Dendritic cell
DCSIGN	DC-specific, ICAM-3 grabbing, non-integrin
DFSOx	Days following onset of symptoms
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DR	Death receptor
ds	Double stranded
DTH	Delayed type hypersensitivity
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein Barr virus
EBV-B-LCL	Epstein Barr virus transformed B lymphoblastoid cell line
EDTA	Ethylene Diamine Tetra Acetic Acid
eIF-5A	Eukaryotic initiation factor-5A
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
ER	Endoplasmic reticulum
E:T	Effector:target
FACS	Fluorescence-activated cell sorting
FADD	Fas-associated death domain
FasL	Fas ligand
FcR	Fc receptor
FCS	Foetal calf serum

FDC	Follicular dendritic cell
FI	Fluorescence index
FLICE	Fas-associated death domain-like interleukin-1beta-converting enzyme
FLIP	FLICE inhibitory protein
$\gamma\delta$	Gamma delta
GALT	Gut-associated lymphoid tissue
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GP	Glycoprotein
HAART	Highly active antiretroviral therapy
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HHV	Human herpes virus
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HPV	Human papilloma virus
HSV	Herpes simplex virus
HTLV-1	Human T cell lymphotrophic virus type 1
i.n.	Intranasal
i.p.	Intraperitoneal
i.v.	Intravenous
ICE	IL-1β converting enzyme
ICS	Intracellular cytokine staining
IFN	Interferon
IL	Interleukin
IRF	Interferon regulatory factor
IU	International units
Jak	Janus kinase
kb	Kilobases
kDa	Kilodaltons
KIR	Killer inhibitory receptor
k/o	Knock-out
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-related herpes virus
LCMV	Lymphocytic choriomeningitis virus
LMP	Latent membrane protein
LTNP	Long term non-progressor
LTR	Long terminal repeat
mAb	Monoclonal antibody
MAC	Membrane attack complex
MBL	Mannan-binding lectin
MCMV	Murine cytomegalovirus
MDC	Myeloid dendritic cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MHR	Major homology region
MHV	Murine herpes virus
MIP	Macrophage inflammatory protein
MLR	Mixed lymphocyte reaction
m.o.i.	Multiplicity of infection
mRNA	Messenger RNA
MV	Measles virus
MVA	Modified vaccinia virus Ankara
MWCO	Molecular weight cut-off
nAb	Neutralising antibody
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor κB

NK	Natural killer
NP	Nucleoprotein
NRTI	Nucleoside reverse transcriptase inhibitor
NNRTI	Non-nucleoside reverse transcriptase inhibitor
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDC	Plasmacytoid dendritic cell
pfu	Plaque forming units
PHA	Phytohaemagluttinin
PIC	Preintegration complex
PKR	dsRNA-dependent protein kinase
PRR	Pattern recognition receptor
PV	Pichinde virus
RANTES	Regulated on activation, normal T cell expressed and secreted
RNA	Ribonucleic acid
rpm	Revolutions per minute
RRE	Rev response element
RT	Reverse transcriptase
rVV	Recombinant vaccinia virus
SAM68	Src-associated in mitosis; 68 kDa
SCID	Severe combined immunodeficiency
SD	Standard deviation
SE	Standard error
SFC	Spot forming cells
SHIV	Simian-human immunodeficiency virus
SIV	Simian immunodeficiency virus
ss	Single stranded
STAT	Signal transducers and activators of transcription
STI	Structured treatment interruption
TAP	Transporter associated with antigen processing
TAR	Transactivation response region
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRADD	TNF receptor-associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
TRANCE	TNF-related activation-induced cytokine
TREC	T cell receptor excision circle
tRNA	Transfer RNA
Tsg101	Tumour suppressor gene 101
UNAIDS	The Joint United Nations Programme on HIV/AIDS
UV	Ultraviolet
vFLIP	Viral FLICE inhibitory protein
VSV	Vesicular stomatitis virus
VV	Vaccinia virus
VZV	Varicella zoster virus
Vβ	V beta
WHO	World health organisation

Table of amino acid symbols

Amino Acid	Single letter code
Alanine	A
Arginine	R
Asparagine	N
Aspartate	D
Cysteine	C
Glutamate	E
Glutamine	Q
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

Chapter 1

Introduction

1.1 HIV: past and present

HIV-1 is a retrovirus in the lentivirus family that causes a persistent infection in humans characterised by progressive failure of host immunological function due predominantly to a selective depletion of helper $CD4^+$ T lymphocytes, and which is ultimately associated with the development of an acquired immune deficiency syndrome (AIDS).

AIDS was first recognised in 1981 when clinicians in New York and California observed among young, previously healthy homosexual men, an unusual clustering of cases of rare diseases. Such cases of Kaposi's sarcoma (KS), opportunistic infections such as *Pneumocystis carinni* pneumonia, and unexplained lymphadenopathy shared a common underlying cause - deficient cell-mediated immunity, resulting predominantly from a significant reduction of circulating $CD4^+$ T cells. Also observed in other diverse risk groups, this syndrome was believed to be caused by an infectious microorganism transmitted by sexual activity or blood; epidemiological studies suggested that the disease might have a retroviral etiology. In 1983 the virus responsible for AIDS was isolated, later to become known as HIV-1 (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984).

It is now evident that cases of AIDS had occurred previously but gone undetected.

Molecular epidemiological studies led to discovery of the origins of the virus. It is believed that the emergence of HIV in the human population was the result of zoonotic transmission. Studies of phylogenetic relationships revealed that HIV-2 (a second type of HIV), first identified in West Africa in 1986 (Clavel, 1986), is closely related to simian immunodeficiency virus isolated from sooty mangabey monkeys (SIV_{sm}), and established the origins of HIV-2 (Gao *et al.*, 1992). HIV-1 was then shown to have probably originated from the Pan troglodytes troglodytes species of chimpanzee carrying SIV_{cpz} (Gao, 1999). Because chimpanzees are killed for food in parts of sub-Saharan Africa, this may have contributed to the species jump.

Since the first reported cases in 1981, up to the end of the year 2003, HIV is estimated to have been responsible for approximately 20 million deaths worldwide. Global estimates made at the end of 2004 (UNAIDS/WHO) revealed that around 39 million people are thought to be living with HIV/AIDS.

A global summary of the epidemic showed that almost 65% of this figure was accounted for by infected individuals living in Sub-Saharan Africa, with a significant proportion of the burden also concentrated in South and South-East Asia. Infection continues to spread at an alarming rate, with almost 5 million new infections in 2004 alone. Intervention is clearly needed to control the spread of this pandemic.

1.2 HIV virology

1.2.1 The basic structure of HIV-1

HIV-1 is an enveloped virus, approximately 110nm in diameter. Each virus particle carries two molecules of single-stranded (ss) linear positive-sense RNA, approximately 9.7kb in length (Figure 1.1).

The envelope is composed of a lipid bilayer, into which are inserted trimers of a virus-encoded glycoprotein (gp). Each glycoprotein spike is composed of three copies of an outer envelope glycoprotein, gp120, each linked to a transmembrane glycoprotein, gp41. Inside the membrane is the matrix protein, p17, within which is found the capsid, composed of p24. Within the capsid is the core. This is a conical electron-dense structure visible in electron micrograph pictures. Inside the core are packaged two copies of the viral genome associated with the nucleocapsid proteins p7 and p9 along with the viral enzymes Protease, reverse transcriptase (RT) and integrase.

The HIV genome contains nine genes, flanked by long terminal repeat (LTR) sequences. Like other retroviruses, HIV has the three major genes, *gag*, *pol* and *env*, which encode structural components and enzymatic proteins. In addition to this, several accessory and regulatory proteins are encoded by the genome.

1.2.2. HIV-1 heterogeneity

HIV-1 is a highly variable virus, and the degree of HIV-1 genetic diversity currently present in the population is far greater than anything that has been described in other human viral infections studied. The genetic diversity of HIV-1 is reviewed in (Gaschen *et al.*, 2002; Lal *et al.*, 2005; McCutchan, 2000; Peeters & Sharp, 2000; Peeters *et al.*, 2003; Robertson *et al.*, 2000).

In the phylogenetic tree of HIV-1 sequences, there are three branches which constitute the M (major), O (outlier) and N groups (non-M, non-O). The three groups are though to represent three independent chimpanzee-to-human transmission events.

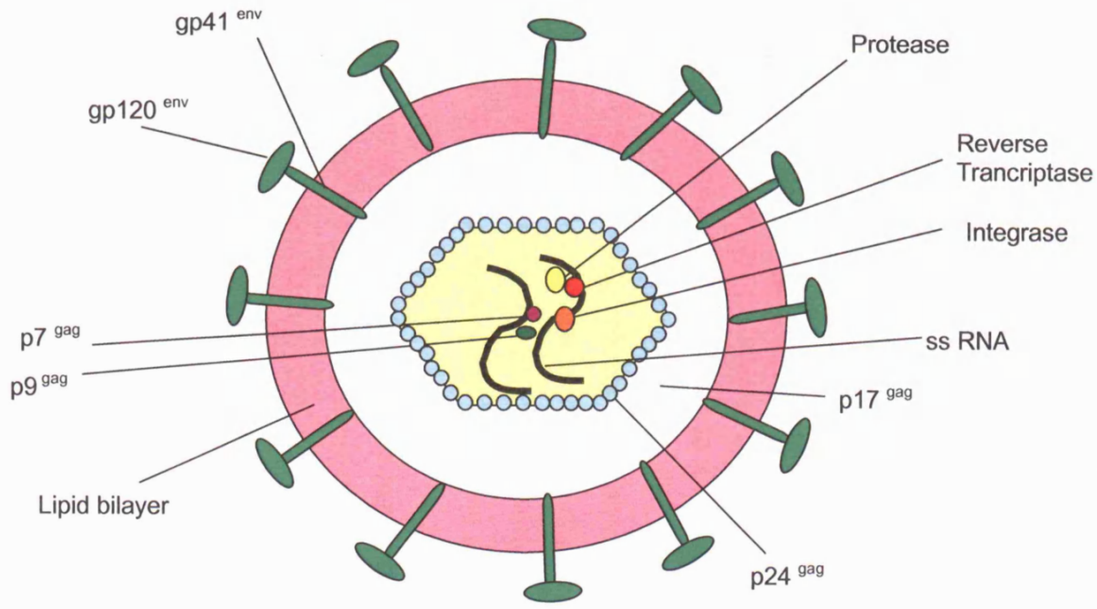


Figure 1.1. Schematic diagram of the HIV-1 virion. The HIV-1 virion is surrounded by a lipid envelope, into which spikes formed of trimers of the two major viral envelope proteins gp120 and gp41, are inserted. Beneath are the products of the gag gene: p24 forms the chief component of the inner shelf of the nucleocapsid, whereas the p17 protein is associated with the inner surface of the lipid bilayer. The retroviral core contains two copies of the single-stranded HIV-1 genomic RNA, which are associated with the nucleocapsid proteins p7 and p9 plus the viral enzymes reverse transcriptase, integrase, ribonuclease and protease.

The group M viruses are by far the most widespread, being the variants of HIV-1 that are responsible for more than 99% of infections worldwide. Intra-group diversification within the human population has led to the formation of distinct genetic subtypes which represent phylogenetically-associated clades of HIV-1 sequences. These are labelled A1, A2, B, C, D, F1, F2, G, H, J and K. The M group subtypes thus represent different lineages, which have some geographical associations. Subtype C is the most prevalent subtype globally, and is common in southern Africa and India. Subtype B is the most dominant subtype in the US and Europe.

Intersubtype recombination creates even more HIV-1 genetic diversity. This may occur when a cell is infected by different viral genomes. The M group therefore also contains a number of recombinant viruses, carrying sections of two or more subtypes in a mosaic genome. When an intersubtype recombinant virus spreads epidemically to establish distinct lineages it is classed as a circulating recombinant form (CRF).

Between clades, up to 30-40% sequence variability can occur, depending on the particular protein under consideration. Sequence variation does not occur evenly throughout the genome. gp120 and Tat are highly variable in comparison to the more conserved gp41 and Gag p24 proteins. Viral strains from the same clade can differ by as much as 5 to 25% in amino acid (aa) sequence (Walker & Korber, 2001), for example by up to 20% in their envelope proteins, but to a lesser extent in the Gag region.

Within an individual, there is further variety within the circulating viral species. During viral replication, the error-prone polymerase generates a genetically diverse quasispecies, a swarm of variants that arises *in vivo*. Replicating viruses can differ as much as 10% in sequence within a single individual (Kuiken *et al.*, 1996; Shankarappa *et al.*, 1999).

From the perspective of vaccine development, the enormous degree of genetic and hence antigenic diversity within HIV-1 represents a formidable challenge.

1.2.3 The HIV-1 life-cycle

The first interaction between HIV-1 and host cells may be a fairly non-specific one mediated by interaction between the virion glycoprotein and glycosoaminoglycans (e.g. heparan sulphate) on the host cell surface. Virion attachment to host cells is mediated by binding of the viral envelope glycoprotein to CD4 (Dalgliesh *et al.*, 1984; Klatzmann *et al.*, 1984), a protein

found on the surface of a limited number of cell types, including T-helper cells and macrophages (Figure 1.2). The HIV envelope then undergoes a conformational change that allows binding to a second cell surface molecule, a co-receptor. The majority of primary HIV-1 isolates use either the chemokine receptor CCR5 as the co-receptor or CXCR4 (the proportion of CXCR4-utilising viruses frequently increases in late-stage infection) (Alkhatib *et al.*, 1996; Deng *et al.*, 1996; Schuitemaker *et al.*, 1992). The gp41 component of the envelope then undergoes a second conformational shift that triggers fusion between the viral membrane and cellular membrane, mediating the entry of the virion contents into the target cell.

Immediately after HIV infects a cell, it uncoats so that the central core of the virion is released into the cell cytoplasm and subsequently disassembled to free the viral genome. The viral reverse transcriptase enzyme, transported with the viral genome, catalyses the synthesis of a linear, double-stranded (ds) cDNA using the viral genomic RNA as a template. The DNA copy of the viral RNA is transported to the nucleus as a preintegration complex (PIC), where it is integrated into host cell DNA by the viral integrase to form a provirus. RNA transcripts produced from the integrated provirus serve as both a source of mRNAs to direct the synthesis of viral proteins and later as RNA genomes for incorporation into new viral particles. The provirus is subject to regulation by cellular transcription factors as well as its own regulatory proteins. T cell activation induces activation of cellular transcription factors such as NFκB, which bind to the viral LTR and initiate low level transcription of the provirus by the cellular RNA polymerase. The first transcripts are processed extensively, producing spliced mRNAs and allowing the translation of early proteins Tat and Rev using the host's ribosomes. Tat acts to amplify transcription from the provirus. Increased levels of Rev allow for the increased transport of singly-spliced or unspliced viral RNAs to the cytoplasm. The unspliced and singly-spliced transcripts encode the late structural proteins. The *gag* and *pol* mRNAs are translated to give polyproteins, long polypeptide chains that are then cleaved by the viral Protease into individual functional proteins. The product of the *env* gene, gp160, has to be cleaved by a host cell protease into gp120 and 41, which are then assembled as trimers into the viral envelope.

New virions are created by assembly of the unspliced transcripts which are to be the new viral genomes with the structural viral components. These are released from the cell by budding through the host cell plasma membrane and undergo subsequent maturation due to the actions of the viral protease.

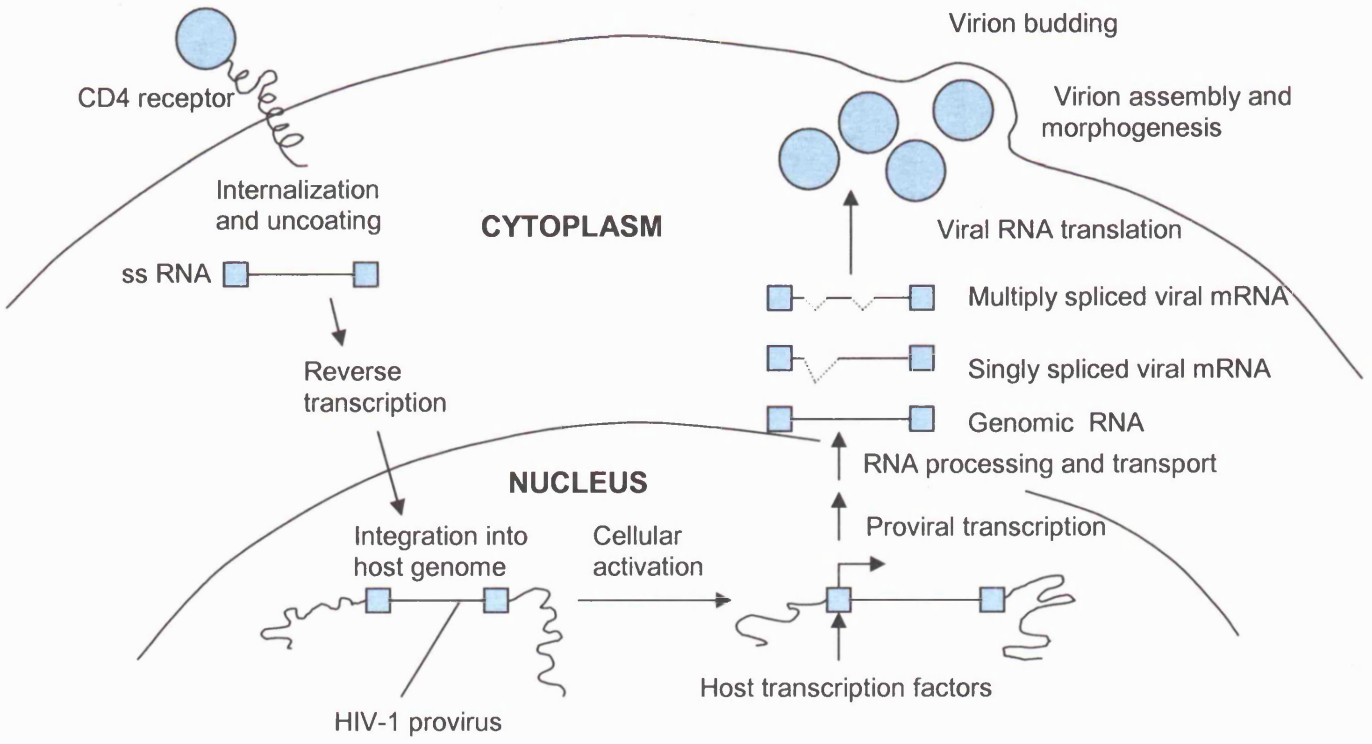


Figure 1.2. Life-cycle of HIV-1. After interaction of gp120 with the CD4 receptor and a chemokine receptor co-receptor, gp41-mediated membrane fusion occurs, and the virion contents enter the cell. After internalisation and uncoating, reverse transcription of viral RNA occurs. This leads to production of a double stranded DNA form of the viral genome. Integrase promotes the insertion of this viral DNA into the cell's genome after the DNA has entered the nucleus, giving rise to the HIV-1 provirus. The expression of HIV-1 genes is stimulated initially by the action of specific host transcription factors with binding sites in the LTR. Their binding leads to the sequential production of various viral mRNAs. The first mRNAs produced correspond to the multiply spliced species encoding Tat, Rev and Nef proteins. Subsequently the viral structural proteins are produced, allowing the assembly and morphogenesis of new virions. The new HIV-1 virions mature budding from the host cell membrane, and can then infect other target cells.

Mature, infectious virions can then re-initiate the life-cycle by infecting other target cells.

1.2.4 The function of HIV-1-encoded proteins

The HIV genome encodes structural (Gag and Env), enzymatic (Pol), plus regulatory (Tat and Rev) proteins and several accessory (Vif, Vpr, Vpu and Nef) proteins (Figure 1.3). The functions of the HIV proteins are briefly reviewed in the following section.

- gp160

The viral glycoprotein gp160 forms part of the viral envelope, and is composed of an external gp120 subunit and a membrane-spanning gp41 subunit. These oligermerize to form trimeric structures on the surface of the virion, composed of six individual subunits, three gp120s and three gp41s. The envelope glycoprotein complex is involved with binding to receptors on target cells and mediating membrane fusion prior to entry into the host cell. The viral glycoprotein can also promote the fusion of infected cells with uninfected neighbouring cells in syncytium formation.

The gp120 and gp41 components of the envelope glycoprotein complex are synthesised as a single co-translationally glycosylated polyprotein precursor, gp160. Folding and assembly into oligomeric structures then occurs (Hunter & Swanstrom, 1990; Willey *et al.*, 1988), which are transported out of the endoplasmic reticulum (ER) to the Golgi complex. In a late Golgi compartment, the precursor is cleaved to gp120 and gp41 by a cellular proteinase. The resulting subunits remain non-covalently associated following cleavage of the precursor, and are transported to the cell surface.

The interactions between gp160 and host cell receptors are complex and involve a series of conformational changes. Both the CD4 binding site and the conserved co-receptor binding site are partially masked by the hypervariable V1/V2 loop structure. Interaction between a gp120 trimer and a cluster of CD4 molecules displaces the V1/V2 loop and the V3 loop, creating the co-receptor binding site (Bandres *et al.*, 1998; Hill *et al.*, 1997; Sattentau & Moore, 1991; Sattentau *et al.*, 1993; Thali *et al.*, 1993; Trkola *et al.*, 1996; Wu *et al.*, 1996) and loosening the attachment of gp120 with gp41. gp120 makes contact with the co-receptor, bringing the virus and cell membranes into close proximity. Further conformational changes then take place involving gp41. gp41 contains a hydrophobic, glycine-rich 'fusion' peptide at the amino terminus that is

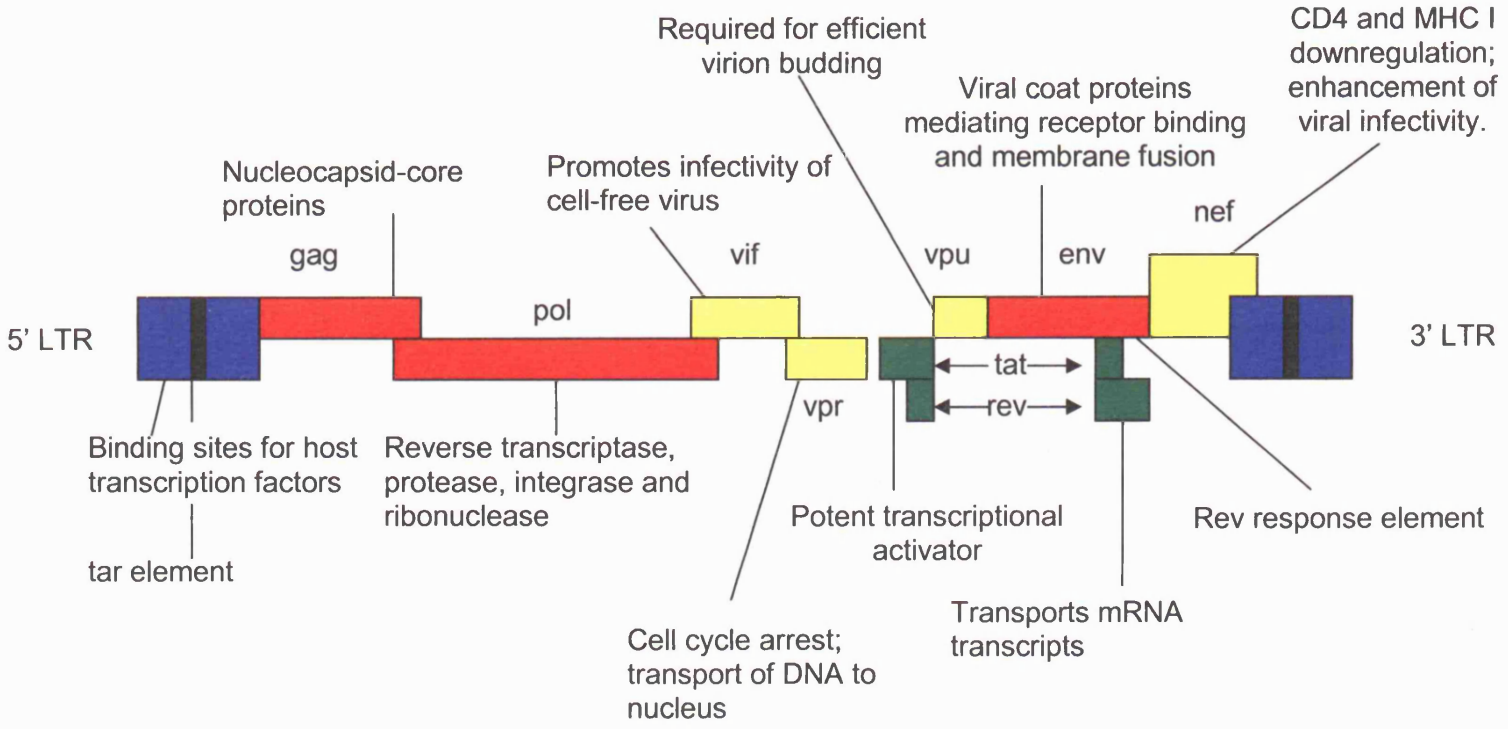


Figure 1.3. Schematic diagram of the HIV-1 genome. The HIV genome contains 9 genes, flanked by LTR sequences. As for other retroviruses, the HIV-1 genome has gag, pol and env coding regions. Regulatory (Tat and Rev) and accessory proteins (Nef, Vif, Vpr and Vpu) are also encoded. The genome can be read in 3 frames and several of the viral genes overlap in different reading frames. This allows the virus to encode many proteins in a small genome. The mRNAs for Tat, Rev and Nef proteins are produced by the splicing of viral transcripts, so their genes are split in the viral genome.

essential for membrane fusion. Upon binding of gp120 to receptors, gp41 undergoes a conformational change from a nonfusogenic, to a trimeric coiled-coil and then a six-helix bundle fusion-active form, exposing the fusion peptide. The transition of gp41 drives membrane fusion through the bringing together of the viral and cell membranes in close enough proximity for the fusion protein to insert into the target cell membrane and fusion to take place (Chan *et al.*, 1997; Weissenhorn *et al.*, 1997). The fusion step of viral entry into host cells is targeted by the fusion inhibitor T20, which prevents the conformational change required for the fusion of viral and cellular membranes (Kilby *et al.*, 1998). Other inhibitors of viral entry (which inhibit gp120 binding to CD4 receptors) have been more recently identified (Lin *et al.*, 2003; Wang *et al.*, 2003) which not only have potential as antiviral drugs, but are also useful as probes to gain further understanding of the HIV-1 envelope structure and viral entry process (Si *et al.*, 2004).

- Gag

The assembly of HIV-1 virions is controlled by Gag, which recruits all of the components needed for formation of a fully infectious virus (reviewed in (Bukrinskaya, 2004)). That Gag drives virus assembly is evidenced by the formation of virus-like particles when Gag is expressed in the absence of other viral proteins (Gheysen *et al.*, 1989).

Gag is a precursor (Gag p55) which is cleaved to give the internal structural proteins of the mature virion (Freed, 1998; Hunter, 1994; Wills & Craven 1991). The viral enzymes are brought into the virion as components of the Gag-Pol polyprotein, produced by ribosomal frame shifting between the overlapping *gag* and *pol* genes.

Following its synthesis, the Gag precursor becomes co-translationally modified through myristylation, which increases its affinity for membranes (Bryant & Ratner, 1990; Gottlinger *et al.*, 1989). Myristylated Gag precursor molecules associate with the inner plasma membrane, where they oligomerise. Eventually a spherical Gag protein shell is formed which protrudes from the cell surface, and a membrane fusion event releases the assembled particle. Budding of virus particles is thought to involve the interaction of Gag complexes with lipid rafts within the plasma membrane (Ono & Freed, 2001). The immature HIV-1 particles which are generated are non-infectious (Gottlinger *et al.*, 1989; Kohl *et al.*, 1988). Subsequently the Gag polyprotein is

processed by the HIV Protease to create four functional mature products: p17, p24, p7 and p6.

The liberated p17 domain remains associated with the lipid envelope, while the p24 and p7 domains condense around the viral genome, giving rise to the characteristic conical core of the mature HIV-1 virion.

During assembly, p17 has well-established roles in the targeting of Gag to the plasma membrane (due to a hydrophobic myristic acid moiety at its N-terminus (Bryant & Ratner, 1990; Gottlinger *et al.*, 1989) and the interaction of a cluster of basic residues at the N-proximal region with acidic phospholipids on the plasma membrane (Zhou *et al.*, 1994)) and in the incorporation of the viral envelope glycoprotein spikes into nascent particles during virus assembly (Dorfman *et al.*, 1994; Freed & Martin, 1995; Yu *et al.*, 1992) (due to interaction between the cytoplasmic domain of gp41 and p17 (Cosson, 1996)). p17 may also have a role in the nuclear import of the HIV-1 pre-integration complex (Bukrinsky *et al.*, 1993; von Schwedler *et al.*, 1994).

p24 has crucial roles in particle assembly and also in uncoating following entry of the virus particle into a new target cell. The N-terminal domain of p24 interacts with cyclophilin A, which leads to the incorporation of this host protein into virions (Franke *et al.*, 1994; Thali *et al.*, 1994), where it may facilitate virion uncoating (Gamble *et al.*, 1996). The C-terminal domain of p24 contains a stretch of 20 residues termed the major homology region (MHR) because it is conserved among unrelated retroviruses (Wills & Craven 1991). The conservation of this region supports for a crucial role of the MHR in virus replication; mutations in this region block virus assembly (Provitera *et al.*, 2001).

The p7 domain contains two zinc finger motifs (Bess *et al.*, 1992) and two clusters of basic residues which are essential for the specific packaging of two copies of the genomic viral RNA into assembling particles (encapsidation) (Berkowitz *et al.*, 1996).

A major function of p6 is to promote the detachment of assembled virions from the cell surface and/or from each other, as evidenced by defects in virus separation in HIV-1 particles lacking p6 (Gottlinger *et al.*, 1991). Viral budding involves the binding of p6 to the host endosomal sorting protein Tsg101 (Carter, 2002; Freed, 2003; Garrus *et al.*, 2001).

- Pol

The Pol gene encodes three enzymes: Protease, RT and integrase. The Pol gene products are initially synthesised as a part of a polyprotein Gag-Pol precursor, $Pr160^{Gag\text{-}Pol}$, and are liberated by autocatalysis of the precursor.

Retroviral proteases are related to cellular aspartic proteases (reviewed in (Davies, 1990)), and their primary role is to mediate the production of mature, infectious virions. The initial cleavage event mediated by the HIV Protease occurs during or immediately after virion release from the cell, and liberates the Protease from the Gag-Pol precursor. Following its release, the enzyme cleaves a number of sites in both Gag and Gag-Pol, leading to conformational changes and a change in virion morphology (maturation), yielding the characteristic condensed cone-shaped core of the virus. The HIV-1 Protease is an important target for antiviral drugs, and inhibition of its function can lead to precipitous declines in viral load (see section 1.8.2).

RT catalyses the conversion of the ss RNA genome into ds DNA during the early stages of the infection (Baltimore, 1970; Temin & Mizutani, 1970). Three steps are involved in this process, all of which are involve RT:

(i) RNA-directed DNA polymerisation, for minus-strand DNA synthesis

(ii) RNaseH activity, for the degradation of the tRNA primer and genomic RNA present in DNA-RNA hybrid intermediates

(iii) DNA-directed DNA polymerisation, for second/plus-strand DNA synthesis

Retroviruses are referred to as psuedodiploid because the genome is packaged into the virion as two copies of ss RNA, but only one provirus is formed per virion (Hu & Temin, 1990). Reverse transcription is dependent upon the 3'-OH group of a primer to initiate polymerisation. Specific tRNAs are employed for this function; HIV-1 utilises $tRNA^{Lys3}$. The high rate of variation among HIV populations is largely a consequence of the error-prone nature of RT as it lacks proofreading activity (Temin, 1993). Along with Protease, RT is also a target for antiviral drugs.

Following reverse transcription, a DNA copy of the viral genome is integrated into the host cell chromosome. This is catalysed by integrase, a 32kDa protein which is composed of three structurally and functionally distinct domains: an N-terminal, zinc finger-containing domain, a core domain and a C-terminal domain. It is the core domain which plays the central role in catalysis.

In the cytoplasm, integrase is involved with the generation of a pre-integration substrate from both strands of the linear viral DNA, giving 3'-recessed ends. In

the nucleus, it catalyses the cleavage of the host DNA, so that the 3'-recessed ends of viral DNA can join to the 5'-overhanging termini of the cleaved cellular DNA. In conjunction with cellular repair machinery, integrase fills in the gaps, thereby completing the integration process (Brown *et al.*, 1989; Fujiwara & Mizuuchi, 1988; Roth *et al.*, 1989). The integrated viral DNA is known as the provirus, and subsequently serves as a template for the synthesis of viral RNA.

- Tat

Although host cellular factors can positively regulate transcription of HIV, the viral Tat protein, acting as a trans-activating factor, greatly enhances the rate of transcription (reviewed in (Strebel, 2003)). It binds to an RNA sequence immediately downstream of the initiation site for transcription in the viral LTR, the transactivation response region (TAR) (Dingwall *et al.*, 1989; Weekes *et al.*, 1990) and markedly increases the efficiency of viral gene expression by augmenting the processing ability of the RNA Pol II transcription complex and stimulating transcription elongation (Kao *et al.*, 1987).

Tat permits activation of the RNA polymerase which, because of its intrinsic instability, disengages from the DNA template prematurely unless Tat is present in the cell. During transactivation, the RNA polymerase carboxyl terminal domain (CTD) is phosphorylated. Following clearance of the promoter, the phosphorylated polymerase is then able to transcribe through the TAR region, synthesising the TAR RNA stem-loop structure which creates a signal for Tat recruitment to the transcription complex. Tat also binds a kinase complex (Herrmann & Rice, 1995), which in the presence of Tat, becomes constitutively activated. This leads to hyper-phosphorylation of the CTD of the polymerase, making the transcription complex more processive, allowing for high levels of HIV transcription (Isel & Karn, 1999). Tat was also found to suppress RT activity during the late stages of viral replication and to increase viral infectivity, presumably by preventing the premature synthesis of DNA (Kameoka *et al.*, 2001).

Apart from its role in activating the transcription of the HIV genome, Tat is associated with additional activities. Because Tat is able to exit HIV-infected cells, some of these effects may be caused by interaction of extracellular Tat with specific cell surface receptors that trigger the activation of signal transduction pathways, or by the uptake of Tat into uninfected bystander cells. Tat may influence cell death, since it was found to cause neurocytotoxicity in

the central nervous system (Kim *et al.*, 2003; Nath *et al.*, 1996; Sabatier *et al.*, 1991) and apoptosis in cultured peripheral blood mononuclear cells (PBMCs) and $CD4^+$ T cell lines (Li *et al.*, 1995; Purvis *et al.*, 1995; Westendorp *et al.*, 1995a; Westendorp *et al.*, 1995b). On the other hand, Tat was found to upregulate Bcl-2 in infected primary human macrophages, suggesting that in some cell types, Tat expression may contribute to cell survival (Zhang *et al.*, 2002b).

- Rev

Rev is a viral protein which regulates the expression and utilisation of viral transcripts through its role in RNA transport.

Eukaryotic cells have mechanisms to prevent export from the nucleus of incompletely spliced mRNA transcripts. This could present a problem for a virus which depends on export of unspliced, singly-spliced and multiply-spliced mRNAs in order to translate the full complement of viral proteins. However, Rev is able to promote the export of unspliced and partially spliced transcripts from the nucleus (Fischer *et al.*, 1995; Wen *et al.*, 1995).

The fully-spliced mRNA transcript encoding Rev is translated early in the infectious cycle. The expressed Rev protein then enters nucleus through an interaction of its nuclear localisation signal with the nuclear import factor importin β (Truant & Cullen, 1999), and binds to a specific viral RNA sequence present in all unspliced and partially spliced mRNAs, the Rev response element (RRE). A nuclear export signal is subsequently exposed which mediates the interaction of Rev with nuclear export factors (Meyer *et al.*, 1996), principally a nucleocytoplasmic transport protein, Crm1, which engages a host pathway for exporting mRNA species into the cytoplasm (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997; Neville *et al.*, 1997). Other host factors involved in Rev-mediated nuclear export include the initiation factor eIF-5A (Elfgang *et al.*, 1999) and SAM68, a protein postulated to play a role in cell cycle control (Li *et al.*, 2002).

Rev is also thought to influence the susceptibility of HIV-infected cells to cytotoxic T lymphocyte (CTL) killing and thereby assist with immune evasion. It was found that an attenuated *rev* allele and variations in Rev activity could downmodulate late gene product expression (namely that of *gag*), and alter the sensitivity of infected cells to anti-Gag CTL killing (Bobbitt *et al.*, 2003). Although the mutation in Rev was introduced artificially, viruses derived from asymptomatic infected individuals also had lower Rev activity, lower Gag

expression and greater resistance to anti-Gag CTL killing. Less active *rev* alleles may therefore be specifically selected by an active immune response because the infected cells harbouring them have a survival advantage in the face of a strong CTL response against late viral genes.

- Vpr

Vpr is packaged into virions through its interaction with the Gag p6 protein (Paxton *et al.*, 1993). It is the only accessory protein present in significant quantities in virions, suggesting that it has important functions during the early phase of infection.

Vpr is thought to have effects on a number of host cellular events including cellular proliferation, differentiation and regulation of apoptosis (possibly via Vpr binding to a protein in the mitochondrial membrane causing mitochondrial membrane permeabilisation and apoptosis (Jacotot *et al.*, 2001)). Vpr is thought to increase viral production in infected cells through arrest of the cell cycle in the G_2/M phase (Poon *et al.*, 1998; Rogel *et al.*, 1995). The HIV LTR is more active in the G_2 phase of the cell cycle (Goh *et al.*, 1998). The influence of Vpr on cellular proliferation and cell cycle arrest may underlie the suppressive effects of Vpr on host antibody and CTL production (Ayyavoo *et al.*, 2002).

Vpr has also been shown to downregulate cytokine and chemokine production, in part through affecting NFκB functions (thereby suppressing host inflammatory responses) (Ayyavoo *et al.*, 1997; Kino *et al.*, 1999; Muthumani *et al.*, 2004; Spiegel *et al.*, 2000). A possible role for Vpr in immune evasion was recently described, where it was reported that Vpr was able to inhibit dendritic cell (DC) maturation and T cell activation by inhibiting the expression of co-stimulatory molecules and cytokines essential for immune activation (Majumder *et al.*, 2005; Muthumani *et al.*, 2005).

Although HIV does not require Vpr for infection of $CD4^+$ T cells, the presence of Vpr is critical for infection in macrophages. One important specific role of Vpr is to facilitate the infection of non-dividing cells by transporting the pre-integration complex into the nucleus (Connor *et al.*, 1995; Hattori *et al.*, 1990; Heinzinger *et al.*, 1994).

Mutations in *vpr* and functionally defective Vpr have been associated with slow disease progression, but further evidence is required to support this correlation (Lunn *et al.*, 2003; Somasundaran *et al.*, 2002; Zhao *et al.*, 2002).

- Vpu

The Vpu gene encodes a small integral membrane phosphoprotein with functions in the induction of proteolytic degradation of newly-synthesised CD4 in the ER (Willey *et al.*, 1992a), and enhancement of viral release from the plasma membrane of infected cells (Schubert *et al.*, 1996). In the ER, Vpu binds to CD4 and targets it for proteolysis in the cytosolic ubiquitin-dependent proteasome pathway (Bour & Strebel, 2000). One consequence of Vpu-mediated CD4 proteolysis is to facilitate the transport and processing of the envelope glycoprotein gp160 (Willey *et al.*, 1992b) which would otherwise be trapped in the ER because of its propensity to form stable complexes with CD4 (Buonocore & Rose, 1990).

Vpu has also been shown to decrease the surface expression of major histocompatibility complex (MHC) class I molecules by interfering with their synthesis, thereby hindering the presentation of HIV antigenic epitopes to HIV-specific CTL (Kerkau *et al.*, 1997).

- Vif

Vif is a gene which is common to all lentiviruses with the exception of equine infectious anaemia virus (Kawakami *et al.*, 1987). It encodes a ~220 amino acid cytoplasmic protein that is essential for virus infectivity (Sodroski *et al.*, 1986b; Strebel *et al.*, 1987).

Vif may play an important role in viral disassembly after infection, the transport of the incoming viral PIC to the nucleus, and provirus formation (Borman *et al.*, 1995; Gabuzda *et al.*, 1994; Gabuzda *et al.*, 1992; von Schwedler *et al.*, 1993; Zhang *et al.*, 2000). Vif is also important for the stability of viral cores (Ohagen & Gabuzda, 2000) and for virus infectivity.

One described indirect role of Vif in enhancing viral infectivity is through its ability to degrade APOBEC3G (apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G), a cellular restriction factor. Cellular restriction factors render retroviruses non–infectious in some species. Vif is able to negate this activity of APOBEC3G (and APOBEC3F). APOBEC3G and APOBEC3F belong to family of cytodine deaminases with RNA-editing activity, involved with post-transcriptional alteration of cellular RNA in which specific nucleotides are catalytically changed. Such alterations may involve the deamination of target bases. APOBEC3G/F induces deamination of the viral cDNA as it is being synthesised, rendering it inactive. In studies using Δvif HIV virions, APOBEC3G/F generates G→ A mutations in reverse transcripts

(Bishop *et al.*, 2004; Harris *et al.*, 2003; Lecossier *et al.*, 2003; Liddament *et al.*, 2004; Mangeat *et al.*, 2003; Mariani *et al.*, 2003; Wiegand *et al.*, 2004; Zhang *et al.*, 2003b; Zheng *et al.*, 2004). These result from the APOBEC-catalysed deamination of cytosines in the template minus strand, resulting in a C→U change. A is subsequently synthesised on the plus strand opposite the U, resulting in G→A switches. The consequences of cytosine deamination for HIV replication are the degradation of most reverse transcripts prior to integration (Mariani *et al.*, 2003) and introduction of stop codons into the open reading frames (ORFs) of the virus through G→ A mutations (Yu *et al.*, 2004). APOBEC3G and APOBEC3F are encapsidated in Δvif HIV virions, but are almost absent in wild-type virions (Kao *et al.*, 2003; Mariani *et al.*, 2003; Marin *et al.*, 2003; Mehle *et al.*, 2004; Sheehy *et al.*, 2003; Stopak *et al.*, 2003; Wiegand *et al.*, 2004; Zheng *et al.*, 2004), suggesting that Vif functions to prevent APOBEC3G and APOBEC3F encapsidation. APOBEC3G is excluded from virions by the actions of Vif, which induces ubiquitination and targets APOBEC3G for destruction in the proteasome (Yu *et al.*, 2003).

- Nef

Nef is a myristoylated protein of 206 amino acids which is expressed early in the viral life cycle. Nef is an important virulence factor (reviewed in (Das & Jameel, 2005)). It has been found to stimulate viral growth in cell culture and *in vivo*, and to be important for achieving high levels of viraemia and for disease induction in rhesus monkeys (Kestler *et al.*, 1991). That it plays a role in enhancing pathogenicity is shown by the presence in non-progressors of attenuated forms of HIV with deletions in regions of *nef* (Deacon *et al.*, 1995; Kirchhoff *et al.*, 1995).

Nef increases viral budding from lipid rafts, which augments viral infectivity, possibly by optimising the maturation process of the virion (Zheng *et al.*, 2001). Nef also has roles in the downregulation of CD4 (Garcia & Miller, 1991) and MHC class I molecules (Schwartz *et al.*, 1996) on infected cells. Nef mediates the downregulation of CD4 by acting as a connector between the receptor and components of the cell protein trafficking machinery, triggering accelerated internalisation of molecules that have reached the cell surface, redirecting some CD4 from the trans-Golgi network to the endosomal compartment, and from the endosome to the lysosome (Aiken *et al.*, 1994; Kim *et al.*, 1999; Lu *et al.*, 1998; Managasarian *et al.*, 1997; Piguet *et al.*, 1998; Piguet *et al.*, 1999; Rhee & Marsh, 1994). Downregulation of CD4 can

enhance HIV replication, for high levels of CD4 on the surface of HIV-producing cells inhibit the infectivity of released virions by trapping the viral envelope (Lama *et al.*, 1999) and can block virion release from infected cells (Ross *et al.*, 1999). The downmodulation of CD4 could also prevent potentially lethal superinfection events (Lama, 2003). Likewise, Nef can also mediate removal of MHC class I molecules from the surface of infected cells, helping to prevent $CD8^+$ T cell recognition (Cohen *et al.*, 1999; Collins *et al.*, 1998; Greenberg *et al.*, 1998; Le Gall *et al.*, 1998; Piguet *et al.*, 1998; Schwartz *et al.*, 1996). Nef also mediates internalisation of CD28, a co-stimulatory molecule necessary for maximal T cell activation (Bell *et al.*, 2001; Swigut *et al.*, 2001). Lower surface levels of CD28 on infected T cells may result in such cells being unable to receive effective co-stimulation following T cell receptor (TCR) engagement, which may be a way for the virus to block T cell activation. Nef is also though to disrupt MHC class II-restricted antigen presentation to $CD4^+$ T cells by downregulating surface expression of MHC class II, thereby inhibiting virus-specific T cell help (Schindler *et al.*, 2003; Stumptner-Cuvelette *et al.*, 2001; Stumptner-Cuvelette *et al.*, 2003).

Nef can also recruit several protein kinases involved in cell signalling and is therefore able to trigger intracellular activation pathways. By intersecting the CD40 signalling pathway in a Nef-dependent manner, HIV induces macrophages to produce and release chemokines which attract resting T cells and stimulate them so that they can support productive viral replication (Swingler *et al.*, 2003). By interacting with signalling proteins acting in the TCR environment and with the TCR-ζ chain, Nef is able to modulate T cell signalling and sensitise T cells to activation via their TCR (Schrager & Marsh, 1999), rendering them more susceptible to efficient viral replication.

Nef is also able to induce upregulation of Fas ligand (FasL) on infected T cells (Xu *et al.*, 1999). The upregulation of FasL expression results in Fas-mediated apoptosis of virus-specific CTL and bystander cells that come into contact with infected cells. In addition, HIV-infected cells themselves are protected from being killed through Fas ligation, as Nef mediates anti-apoptotic effects. Nef blocks the induction of apoptosis via both Fas and TNF (tumour necrosis factor) receptor-mediated death signals. These require activation of apoptosis signalling regulating kinase 1 (ASK-1), the activity of which is blocked by Nef (Geleziunas *et al.*, 2001). Nef is also able to phosphorylate Bad, a pro-apoptotic molecule, resulting in a block in apoptosis (Wolf *et al.*, 2001). HIV-1

infected cells are also protected from p53-mediated apoptosis due to the destabilisation of p53 upon interaction with Nef (Greenway *et al.*, 2002).
In summary, Nef-mediated signalling is able to support viral replication and enable viral survival through a variety of different mechanisms.

1.3 The course of HIV-1 infection

The course of HIV infection can be divided into three stages (Figure 1.4):
a) Primary infection, characterised by an acute burst of viral replication, accompanied by a transient drop in the drop in the number of circulating CD4 T cells. The acute viral burst typically occurs after a 2-4 week incubation period, and in ~70% of individuals is symptomatic (symptoms typically including fever, rash, oral ulcers and lymphadenopathy) (Cooper *et al.*, 1985; Kinloch-de Loes *et al.*, 1993). Seroconversion usually takes place as the acute viral burst is contained.
b) An asymptomatic period, during which viral replication continues, accompanied by a gradual and progressive decline in the number of circulating $CD4^+$ T cells and impairment in helper T cell (Th) function. The duration of this period varies and may be prolonged in some individuals.
c) The late phase, which is preceded by an increase in viral replication and a sharper drop in $CD4^+$ T cell numbers. This stage is characterised by very low CD4 counts and an increased incidence of opportunistic infections and tumours associated with end-stage AIDS disease, culminating in death.

(a) Events during primary infection

HIV-1 infection generally occurs across a mucosal surface and/or following virus transmission into the blood. The main route of spread is by sexual contact; other important routes include exposure to contaminated blood and blood products and vertical spread from mother to child. Transmission is dose-related, with a direct relationship between the viral load in peripheral blood and frequency of transmission (Gray *et al.*, 2001b; Quinn, 2000).
After the virus enters a host, a sequence of events leads to its dissemination around the body. The virus is infectious either as a free particle or in association with virus-infected cells. Once HIV crosses the mucosal barrier (in the case of sexual transmission), the virus makes initial interactions with host cells and local amplification of infection occurs. Dissemination to draining lymph nodes follows, and then the systemic dissemination of virus and infected cells, leading to an acute burst of viral replication, much of which is

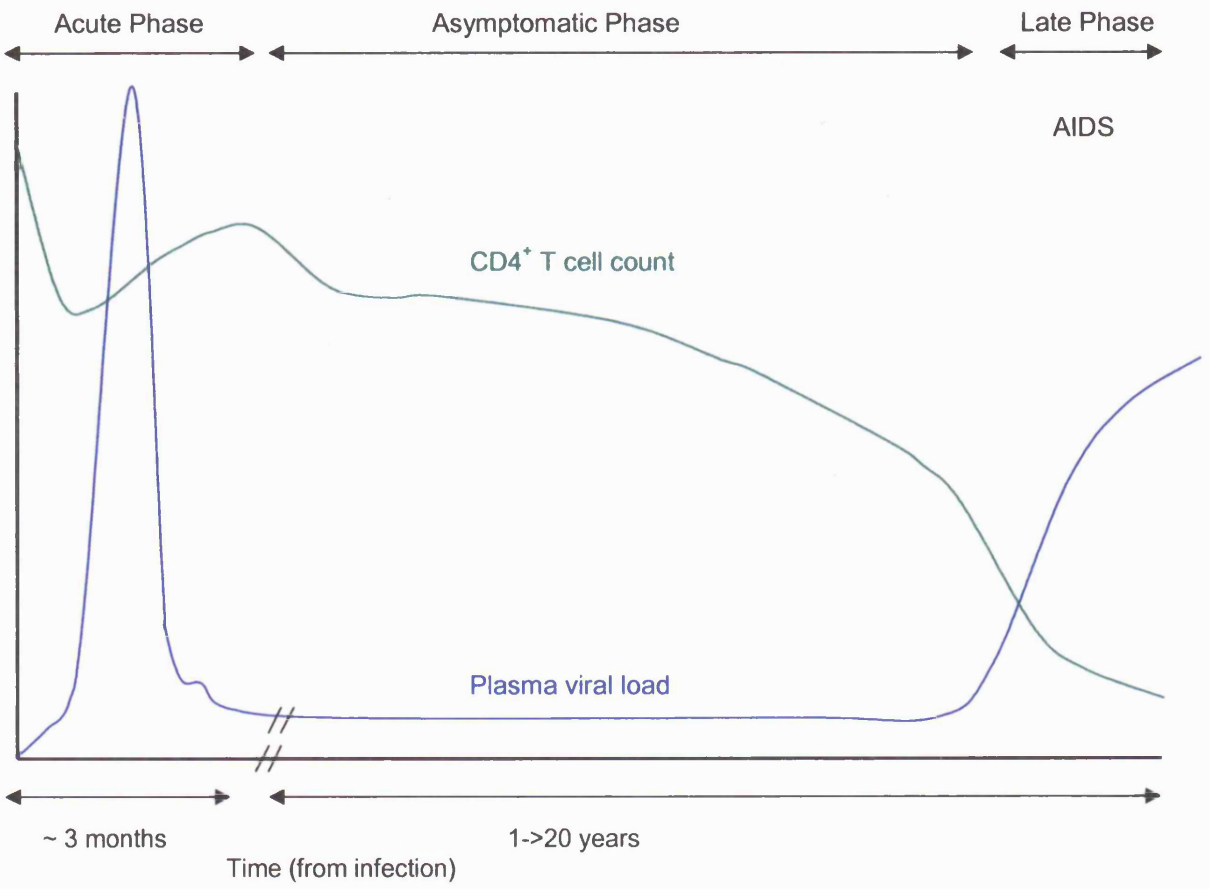


Figure 1.4. Diagram to illustrate the typical course of HIV-1 infection. HIV-1 infection can be divided into three phases. During the acute phase an acute burst of viral replication occurs, accompanied by a transient decline in the $CD4^+$ T cell count, after which viral replication is contained to a setpoint level of persisting virus. The asymptomatic phase is a period of continued viral replication and gradual depletion of $CD4^+$ T cells. In the late phase of infection there is an increase in the level of viral replication accompanied by a more dramatic drop in the $CD4^+$ T cell count. This is associated with AIDS-defining illnesses which eventually result in death.

thought to occur in memory $CD4^+$ T cells in gut-associated and other lymphoid tissues (Li *et al.*, 2005; Mattapallil *et al.*, 2005).

After the virus is transported across the mucosal barrier, local propagation of the virus can occur through the infection of $CD4^+$ T cells, macrophages, and possibly also DCs. Although DCs may constitute a cellular site of productive viral replication, DCs are thought to be more important in the initial capture of HIV in mucosal tissues, and the transport of virions into lymphoid tissues. A surface glycoprotein, DC-specific ICAM-3 grabbing non-integrin (DC-SIGN), expressed by DCs enables these cells to bind to HIV gp120, capturing HIV without infection occurring (Geijtenbeek *et al.*, 2000). Virions bound to DC-SIGN can then be passed from DCs to $CD4^+$ T cells with which they interact.

Macrophages are also thought to be important in enhancing viral spread, owing to the actions of HIV Nef. Through mechanisms involving the CD40 signalling pathway, macrophages release a paracrine factor that renders T cells permissive to infection (Swingler *et al.*, 2003), thereby facilitating the infection of resting T cells and promoting viral spread.

The majority of viral amplification during acute infection has been found to occur in memory $CD4^+$ T cells (both resting and activated). The extent of infection is significant, with 30-60% of memory $CD4^+$ cells throughout body estimated to be infected by virus at the peak of SIV infection (Mattapallil *et al.*, 2005). It has recently become appreciated that, in accordance with the degree of infection, there is a tremendous loss of $CD4^+$ T cells from lymphoid tissues (especially the gut-associated lymphoid tissue (GALT)) during primary infection (Li *et al.*, 2005; Mattapallil *et al.*, 2005). The loss of $CD4^+$ cells could either be due to the direct killing of infected cells and/or may occur as an indirect consequence of viral replication (i.e. the bystander death of cells not infected with virus):

- virus-induced destruction

HIV replicates preferentially in memory $CD4^+$ cells (at least 99% of circulating virus is produced by infected memory $CD4^+$ T cells) which consequently die. The half life for infected, virus-producing $CD4^+$ cells is thought to be less than one day (Markowitz *et al.*, 2003). The death of HIV-infected cells can result from the tendency of infected lymphocytes to form short-lived syncytia or from increased susceptibility of cells to death. Specific HIV proteins may mediate these effects. For example, Env, by binding nascent CD4/coreceptor proteins

in the ER, may disrupt membrane integrity through the exposure of envelope fusogenic domains (LaBonte *et al.*, 2000; Lifson, 1986; Sodroski *et al.*, 1986a). Vpr is able to activate caspases and induce host cell apoptosis (Somasundaran *et al.*, 2002; Stewart *et al.*, 1997). In addition, virus-infected cells may be destroyed by host immune effector mechanisms, for example as a result of the lytic activity of natural killer (NK) cells or virus-specific $CD8^+$ CTL.

- Bystander death/immune activation

In addition to infected $CD4^+$ T cells being destroyed as a consequence of infection, both infected and uninfected cells may potentially be destroyed in a bystander fashion as a consequence of the immune activation occurring during HIV infection. HIV induces a state of chronic immune activation due to the massive antigen stimulation by HIV plus antigen-independent stimulation by cytokines produced by activated antigen presenting cells (APCs) and T cells. Such immune activation can drive further virus replication through the generation of activated CD4 cells, which are good targets for virus replication. However, such chronic activation is associated with deleterious effects on the host, for example it may induce bystander apoptosis of enormous magnitude. This may occur non-specifically owing to the activated APC and cytokine-rich environment associated with immune activation and subsequent activation-induced cell death. In addition, viral proteins may contribute to bystander apoptosis. Nef upregulates FasL on infected cells (Geleziunas *et al.*, 2001). Activated Fas-expressing CD4 cells and CTLs may undergo bystander apoptosis after interacting with FasL on infected CD4 T cells. Env has also been implicated in the initiation of Fas-mediated apoptosis in SIV infection (Li *et al.*, 2005).

Evidence suggests that both mechanisms of CD4 cell depletion may be important in primary HIV infection. Direct viral infection was reported to account for the bulk of the CD4 cell depletion in one study (Mattapallil *et al.*, 2005), but another suggested that only a proportion of memory CD4 cell depletion could be accounted for by direct infection, and that extensive levels of indirect depletion could occur through Fas-mediated apoptosis (Li *et al.*, 2005).

In support of the latter study, it has been shown by in situ labelling of lymph nodes of HIV and SIV-infected subjects that apoptosis occurs predominantly in

bystander cells and not in the productively infected cells themselves (Finkel *et al.*, 1995).

The massive viraemia that occurs during primary infection is transient (Daar *et al.*, 1991). The virus is then contained (in as little as 10 days). Part of the decrease in viraemia is probably due to the loss of susceptible target cells (Phillips, 1996). However, because the containment of the acute viral burst is concomitant with the induction of antiviral immune responses, the rapid and spontaneous decline in the large viral burden during the primary phase of infection suggests an effective immune response in the host (Clark *et al.*, 1991). However, immunological control is only partial; the virus is not completely cleared and, instead, a steady state of ongoing viral replication, associated with a 'stable' viral RNA load (termed the setpoint viral load) is reached and a chronic infection is established.

The clinical course of disease widely varies among different individuals (Haynes *et al.*, 1996). The median time between the acquisition of HIV and development of AIDS is around 10 years. However, ~10% of HIV-infected individuals progress to AIDS within 2-3 years of seroconversion and are classified as rapid progressors. At the other end of the spectrum, 5-10% of HIV-infected individuals remain asymptomatic with stable CD4 counts for at least 10 years after seroconversion, and are classified as non-progressors.
The setpoint viral load established during primary infection is thought to be a prognostic indicator of subsequent disease progression (Lyles *et al.*, 2000; Mellors *et al.*, 1996): the higher the setpoint plasma RNA load, the faster the loss of $CD4^+$ T cells and the shorter the duration of infection before death (Figure 1.5). This highlights the importance of events that take place during the early stages of infection. These early events are affected by multiple factors, including the virulence of the virus isolate, host genetic factors and host antiviral immune responses.

Less pathogenic viruses (such as those with attenuating *nef* mutations) are more readily controlled and are associated with non-progression (Deacon *et al.*, 1995; Kestler *et al.*, 1991; Kirchhoff *et al.*, 1995), highlighting how the intrinsic pathogenicity of the virus can influence the disease course.
The genetics of the host can determine whether infection even occurs, and then if so, can influence the rate of disease progression. For example,

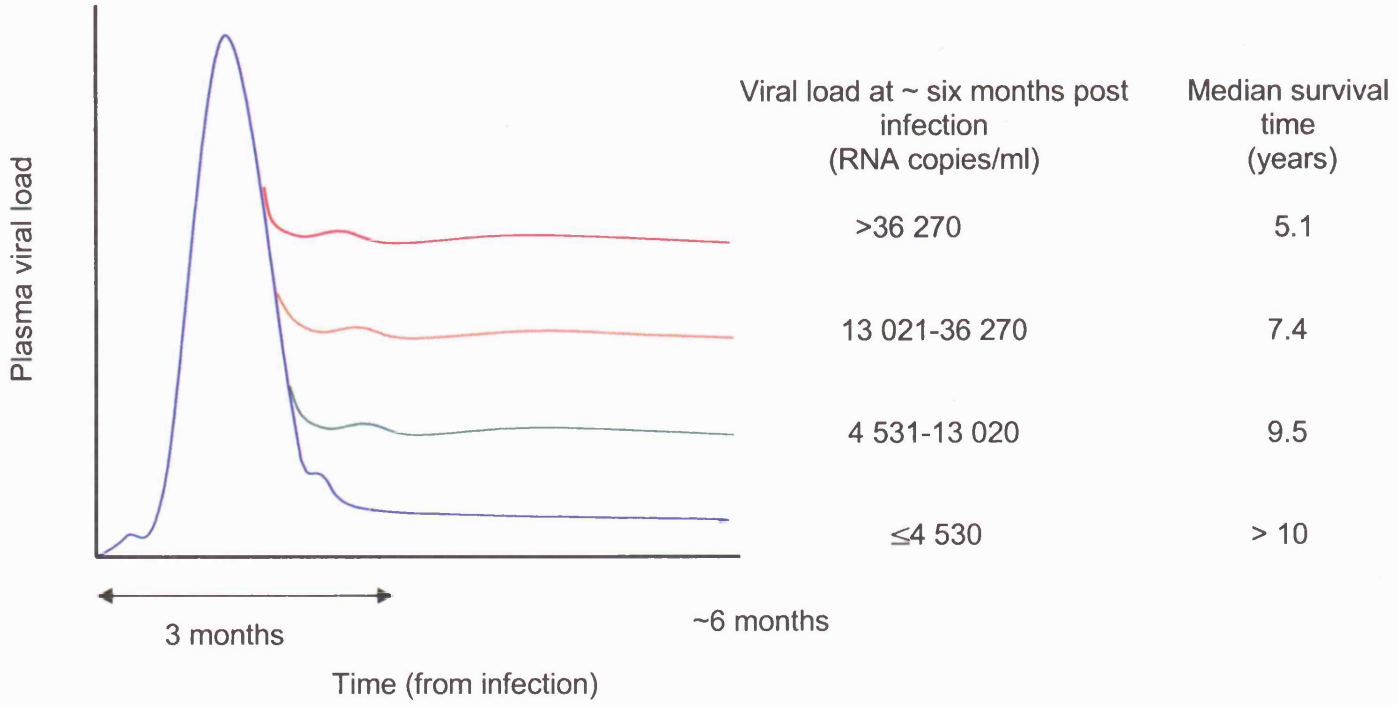


Figure 1.5. The setpoint viral load established at ~six months post-infection is predictive of the subsequent disease course. The level of persisting virus established at ~six moths post-infection (the setpoint viral load) has been shown to be a prognostic indicator, predicting the time to development of AIDS. Patients can be divided up into four different quartiles on the basis of their setpoint viral load; these are schematically represented in the diagram above. The viral load range of each quartile is given, along with the estimated survival time of patients falling into each of the four quartiles (data from Mellors *et al.*, 1996).

individuals homozygous for a deletion of 32 base pairs in the CCR5 gene (~1% of white populations) are extremely resistant to HIV infection (Dean *et al.*, 1996; Liu *et al.*, 1996; Samson *et al.*, 1996), and an association has been found between CCR5 genotype and disease progression rate (Balfe *et al.*, 1998). Many of the genes that influence the course of infection probably do so via affecting immune control of virus replication. For example, the human leucocyte antigen (HLA) type of the individual can influence the course of infection, as discussed later (Carrington & Bontrop, 2002; Carrington & O'Brien, 2003; O'Brien *et al.*, 2001; Roger, 1998); HLA-B alleles are thought to have a particularly dominant influence (Kiepiela *et al.*, 2004). The role of host antiviral immune responses in control of HIV replication is discussed in section 1.6.

(b) The asymptomatic stage of HIV infection

After the acute viral burst, HIV replication continues at a lower level, predominantly within activated $CD4^+$ T cells. HIV preferentially infects and eliminates HIV-specific CD4 cells (Douek *et al.*, 2002) (further compromising the virus-specific immune response), but there is also a general loss of activated CD4 cells which, as in the primary phase of infection, likely reflects a combination of death of infected cells and bystander cell destruction.

The continuing replication of HIV during the asymptomatic period is associated with the rapid turnover of CD4 cells. It is estimated that at least 10^{10} virions are produced each day, and that infected CD4 cells have a half-life of less than a day (Ho *et al.*, 1995; Markowitz *et al.*, 2003; Wei *et al.*, 1995).

Much of the data on viral dynamics were calculated from studies of the effects of highly active antiretroviral therapy (HAART) on the viral load and CD4 count in treated patients. After initiation of HAART there is an initial rapid decline in viral load (due to antiviral effects on virus produced predominantly from activated CD4 cells), and then slower phases of decline reflecting different viral reservoirs which decay with differing kinetics. Follicular dendritic cells (FDCs) represent one such stable reservoir. FDCs harbour trapped stores of virus which remain infectious for long periods (Smith *et al.*, 2001), facilitating the transmission of infection to cells migrating through lymphoid follicles (Embretson *et al.*, 1993). HIV also maintains stable reservoirs by establishing latent infection in quiescent (G_0) T cells. The activity of integrated viral genomes is influenced by the metabolic and activation state of the host cell, and so in such quiescent cells the HIV provirus is not transcribed and can

persist in a latent form until their activation (Chun *et al.*, 1997; Finzi *et al.*, 1997; Wong *et al.*, 1997). It is the long-lived sources of virus represented by FDCs and latently infected cells which are particularly resistant to eradication by HAART and make it difficult to eliminate the virus. The big increase in CD4 cell numbers in the periphery after HAART is thought to be due in part to redistribution of cells from lymphoid tissues to the blood (Bucy *et al.*, 1999; Pakker *et al.*, 1998). When APCs initiate the induction of immune responses within lymphoid organs, $CD4^+$ T cells are stimulated and retained within them (whilst activated $CD8^+$ T cells move into the circulation) (Bishop *et al.*, 1990; Bujdoso *et al.*, 1989). This leads to an apparent decrease in the proportion of $CD4^+$ T cells in the peripheral circulation (Grossman *et al.*, 1993; Rosenberg *et al.*, 1993), which is redressed when viral loads and immune activation are reduced by HAART.

The peripheral $CD4^+$ T cell pool can normally be replenished by production of new T cells from the thymus and by extrathymic proliferation of mature T cell subpopulations. Interleukin (IL)-7 has been shown to be able to stimulate proliferation of progenitor cells and mature T cell subpopulations and inhibit apoptosis to mediate T cell homeostasis (Schluns *et al.*, 2000). IL-7 therefore stimulates increased T cell production both in the general setting of lymphopenia (Schluns *et al.*, 2000) as well as in the context of HIV infection (Fry *et al.*, 2001; Napolitano *et al.*, 2001) (although IL-7 can actually exacerbate infection by driving the division of cells in which the virus can efficiently replicate and also by acting as a cofactor in transactivation of the viral LTR (Chene *et al.*, 1999)).

However the attempts of such compensatory feedback mechanisms to replenish the peripheral $CD4^+$ T cell pool by production of new T cells from the thymus (and by proliferation of mature T cell subpopulations) are frustrated by impairment of thymic function in HIV infection. Using the number of TCR excision circles (TRECs) as a measurement for T cell output by the thymus, a decrease in the thymic production of naïve T cells was observed (Douek *et al.*, 1998). This may reflect two direct effects of the virus: the destruction of progenitor cells from which naïve CD4 cells can be produced and disruption of the thymic microenvironment (the integrity of which is important for T cell maturation).

Therefore, although initially, homeostatic mechanisms act to stop $CD4^+$ cell numbers falling, the production of naïve $CD4^+$ T cells does not keep pace with

$CD4^+$ T cell destruction over time, and $CD4^+$ T cell numbers gradually decrease.

In summary, during the period of asymptomatic infection, a high level of ongoing viral replication continues. This causes a gradual depletion of $CD4^+$ cells not only by direct infection and killing of cells, but also through indirect effects of viral replication on uninfected cells. Over time, the rate of $CD4^+$ T cell destruction exceeds that of the ability to replace them due to impaired production of new T cells, and peripheral $CD4^+$ numbers fall, eventually declining below the level required for normal immune function, leading to the end stage of AIDS disease (Douek, 2003; McCune, 2001).

(c) Late stage infection

When $CD4^+$ T cell numbers decline below a critical level, immune responses that require effective T cell-mediated immunity for host defence are compromised. The profound immunosupression in AIDS patients therefore leads to increased susceptibility to a variety of opportunistic infections including those by *Pneumocystis carinii* and mycobacteria. AIDS patients are also susceptible to malignancies, such as Kaposi's sarcoma, a cancer associated with infection with human herpes virus (HHV) -8. With the occurrence of tumours, opportunistic infections and/or wasting/central nervous system (CNS) disease in late stage infection, untreated HIV-1 infection is almost invariably fatal.

1.4 Overview of antiviral immune responses

Infection of a host by a pathogen induces both an innate and an adaptive immune response. The innate response is the first line of defence encountered by a pathogen. It is mounted rapidly to deal with an infection, being activated in response to conserved microbial patterns which are characteristic of pathogens but not mammalian cells, rather than specific antigens. For example, dendritic cells and macrophages express pattern recognition receptors (PRRs) such as the toll-like receptors (TLRs) (Akira & Hemmi, 2003), and lectin-type receptors. Upon the detection of the presence of a foreign pathogen, intracellular signalling pathways are activated by the PRRs and a cascade of processes are initiated, resulting in the activation of transcription factors, and the induction of cytokines which mediate effector and immunomodulatory roles in the early stages of infection. Other cell types

involved in the innate immune response include NK cells, NK-T cells & gamma delta ($\gamma\delta$) T cells. The adaptive immune response, comprising a humoral, antibody-mediated response, and a T cell mediated response, is more slow to evolve, but targets infections in an antigen-specific manner. Further, the adaptive response possesses immune memory, which may confer long lasting protection and/or enable a more rapid secondary response to be mounted on re-encounter with the pathogen. The different arms of the immune system operate in a co-ordinate manner, with cells and soluble mediators of the innate and adaptive responses interacting to orchestrate an antiviral response that will deal with the pathogen efficiently, and either contain or eliminate it.

1.4.1 Innate immunity

1.4.1 (a) Type I interferons (IFNs)

The IFN-α subtypes, IFN-β and several other IFN species make up the family of type I interferons, cytokines that are rapidly produced in response to infection and which have important antiviral effector roles, in addition to key immunoregulatory functions (Muller *et al.*, 1994). Type I IFNs can be synthesised by many cell types when they are infected. However, the major producer of type I IFNs in most infections is the plasmacytoid dendritic cell (PDC), a precursor to the type 2 DC (reviewed in (Santini *et al.*, 2002; Tough, 2004)). Such cells produce 200-1000 times more IFN than other blood cells after microbial challenge (Siegal *et al.*, 1999). PDCs can be triggered to produce type I IFNs following recognition of viral components via TLRs (Akira & Hemmi, 2003). PDCs express particularly high levels of TLR-7, which recognises single stranded RNA (e.g. influenza virus and vesicular stomatitis virus (VSV) RNAs (Diebold *et al.*, 2004; Lund *et al.*, 2004)) and TLR-9, which recognises double stranded viral DNA (e.g. herpes simplex virus (HSV) -2 DNA (Lund *et al.*, 2003)). Glycoproteins on viral envelopes can be also be recognised (by lectin receptors) to induce IFN production (Ito, 1994). The requirement for type I IFNs for control of virus infections has been demonstrated using type I IFN-R deficient mice; these animals are unable to control infection even with a low dose of viruses such as lymphocytic choriomeningitis virus (LCMV) (Muller *et al.*, 1994; van den Broek *et al.*, 1995). The type I IFNs mediate their effects through binding to receptors that are broadly expressed on many cell types, and signalling the induction of many different genes. These may contribute to antiviral defence indirectly, for example IFNs increase MHC class I expression, and mediate activation of NK

cells, or directly, by conferring resistance to viral replication on the responding cell. IFNs are also important in linking innate and adaptive immunity, since IFNs can promote the activation and differentiation of DCs to stimulate Th1 responses and cross-prime $CD8^+$ T cell responses (reviewed in (Santini *et al.*, 2002)).

1.4.1 (b) Dendritic cells

DCs are resident in most tissues throughout the body, and play a prominent role in pathogen surveillance and the orchestration of both innate and adaptive immune responses.

DCs were initially identified as potent APCs, specialised in capture of antigens at sites of infection, migration to secondary lymphoid organs and T cell priming, having the unique ability to mediate the activation of naïve antigen-specific T cells (reviewed in (Bancherau & Steinmann, 1998; Banchereau *et al.*, 2000)). However it is now recognised that they have a central immunoregulatory role in the adaptive response, being involved in the maintenance of peripheral tolerance in addition to the triggering of antigen-specific T cell responses (Steinman *et al.*, 2003), and functioning not only to initiate adaptive responses, but also to direct the nature of the response (e.g. type 1 or type 2-biased) that is induced (Kapsenberg, 2003). Further, they have been shown to serve both an effector role (via production of cytokines such as type I IFNs) and a regulatory role (e.g. modulating NK cell activity (Andrews *et al.*, 2003; Ferlazzo *et al.*, 2002; Gerosa *et al.*, 2002)) in the innate response.

These diverse functions are performed by heterogeneous DC subpopulations; for example in human peripheral blood, $CD123^+$ PDCs (which as discussed above, act as a potent source of type I IFN production) and $CD11c^+$ $CD123^{dim}$ myeloid DCs (MDCs) are two of the principal DC subsets. The functional properties of DC subsets are determined in part by their developmental lineage and in part by the maturation and activation signals they receive – hence DCs act as a medium via which signals from the local environment can be translated into initiation of an appropriate type of immune response (Shortman & Liu, 2002). DCs are highly responsive to stimuli such as microbial infection, inflammation and tissue damage (Reis e Sousa, 2001); and their maturation is further driven by interaction with T cells. As discussed below, one of the ways in which $CD4^+$ T cells provide help for the induction of

$CD8^+$ T cell responses is via DC activation (Bennet *et al.*, 1998; Ridge *et al.*, 1998; Schoenberger *et al.*, 1998).

1.4.1 (c) NK cells

NK cells are non T cell lymphocytes ($CD3^-$) which make up ~15% of the lymphocyte pool and typically express the marker CD56 in humans (Cooper *et al.*, 2001). Two functionally and developmentally distinct NK cell subsets can be distinguished on the basis of their cell surface density of CD56 expression. ~90% of NK cells fall into the $CD56^{dim}$ subset. These cells are more cytotoxic and express higher levels of FCγRIII/CD16 than the $CD56^{bright}$ subset (Cooper *et al.*, 2001). Consequently they can ligate antibody-coated targets more efficiently and mediate greater levels of antibody-dependent cell-mediated cytotoxicity (ADCC). $CD56^{bright}$ NK cells are hypothesised to have a more immunoregulatory role.

NK cells play an important role in innate defence as they can lyse infected cells (via perforin-mediated effects) and provide an early source of effector and immunoregulatory cytokines. Cytokines and chemokines produced by NK cells include IFN-γ, TNF-α, IL-10, IL-13, macrophage inflammatory protein (MIP) 1α, MIP1β and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Fehniger *et al.*, 1999) (Aste-Amezaga *et al.*, 1994) (Colucci *et al.*, 2003). NK cell activity is enhanced by innate cytokines such as IL-12, IL-18 and type I IFNs (Biron & Brossay, 2001). Their effector functions are controlled by the balance of signals received via surface receptors which fall into two types: activatory receptors and inhibitory receptors (Leibson, 1997) (Lanier, 2001). Activatory receptors (such as NKG2D) associate with adaptor molecules which bear activating motifs associated with signalling pathways which function in NK cell activation. By contrast, inhibitory receptors (which include the killer cell immunoglobulin-like receptor (KIR) family and the CD94-NKG2A heterodimers) have inhibitory motifs in their cytoplasmic domain which prevent NK cell functions. The importance of NK cell function in some viral infections is unclear (e.g. LCMV, (Welsh, 1996)); but is known to be particularly important in control of primary herpersvirus infections (Biron *et al.*, 1989; Biron *et al.*, 1999; Bukowski *et al.*, 1985).

1.4.1 (d) NK-T cells and $\gamma\delta$ T cells

NK-T cells and $\gamma\delta$ T cells are minor lymphocyte subsets which express only a limited diversity of receptors. Both behave as intermediates between innate

and adaptive immunity. Like NK cells, NK-T cells and $\gamma\delta$ T cells perform anti-viral functions by direct lysis of infected cells, or through the production of cytokines and chemokines that interfere with viral transmission and/or replication or that activate other effector cells (Lang *et al.*, 1995).

NK-T cells have phenotypic properties of both NK cells and T cells, expressing both NK cell markers and T cell receptor molecules. Unlike T cells, NK-T cells have a restricted T cell receptor repertoire, and are stimulated as a result of presentation of a non-peptide antigen (α-GalCer) by the non classical-MHC class I-like molecule CD1d. In humans, NK-T cells have an invariant α chain (Vα24) and most express Vβ11. They play an important regulatory role in a number of immune responses. Like NK cells, NK-T cells can also recognise targets deficient in expression of MHC class I molecules (Poccia *et al.*, 2001).

$\gamma\delta$ T cells are a subset of T cells that express CD3 and $\gamma\delta$ rather than $\alpha\beta$ TCRs. They also recognise non-peptidic antigens without MHC restriction. The repertoire of $\gamma\delta$ T cell receptors is relatively limited; at least some $\gamma\delta$ T cells can be activated *in vitro* with nonpeptidic phosphorylated molecules of mycobacterial origin, to induce cytotoxicity and TNF production (Lang *et al.*, 1995). Although $\gamma\delta$ T cells constitute a minor population of T cells in lymphoid tissues, a high proportion of T cells in epithelial tissues express $\gamma\delta$ receptors.

1.4.1 (e) The complement system

The complement system comprises a series of interacting complement proteins which are sequentially activated from an inactive to an active form in an enzyme-driven amplification cascade. The activation of complement can occur by way of three pathways:

i) The classical activation pathway which involves the binding of the complement protein C1q to antigen-antibody complexes or directly to virions.

ii) The mannan-binding lectin (MBL) pathway, where MBL binds to oligosaccharides on pathogen surfaces.

(iii) The alternative pathway, which involves deposition of the complement factor C3b on the surface of host cells or pathogens.

Activation of any of these pathways results in cleavage and activation of C3, followed by cleavage of C5 and further downstream events which culminate in the formation of membrane attack complexes (MAC). Assembly of the membrane attack complex on the surface of an infected cell or lipid-enveloped virus generates a pore in the lipid bilayer membrane which disturbs the

osmotic potential, and results in the eventual destruction of the cell or pathogen.

The potentially destructive effects of uncontrolled complement activation are avoided by the activity of complement-regulators (e.g. CD46, CD55 and CD59) which act to modulate the complement activation cascade by dissociating complexes or catalyzing the enzymatic degradation of covalently bound complement proteins.

Complement activation does not contribute to control of viral infections only by way of MAC-mediated destruction of infected cells or enveloped viruses. Opsonisation of virions and infected cells by the deposition of C3b and subsequent uptake by complement receptor (CR) bearing cells may lead to phagocytic removal of the pathogen. The production of various activated complement proteins can also result in recruitment of antibody, complement and leucocytes to the site of infection. Furthermore, interaction of complement components with virion surfaces in itself has been reported to inhibit the infectivity of certain viruses (e.g. C1q and human T cell lymphotrophic virus type 1 (HTLV-I) or MBL and influenza A).

1.4.2 The adaptive immune response

Each arm of the adaptive immune response has an important role in controlling viral infections, but the relative contributions of each may vary according to the infection. This can be revealed by depletion and adoptive transfer studies in mice, or inferred in human infections by study of patients with selective immunodeficiencies. For example, patients with hypogammaglobulinaemia (a defect in antibody production), have an increased risk of infection with enteroviruses, highlighting the importance of antibody-mediated immunity during infection with these viruses (Sanna & Burton, 2000). By contrast, people with T cell deficiencies are prone to infection with cytomegalovirus (CMV), HSV, varicella zoster virus (VZV) and measles virus (MV) indicating that T cell-mediated immunity is critical to containing infection with these viruses (Borrow & Oldstone, 1994). Although particular arms of the adaptive immune response may play a more dominant role in control of different virus infections, the synergistic interaction between different components of the adaptive immune response is often key to an effective immune response (Doherty *et al.*, 2001; Marten *et al.*, 2001; Thomsen *et al.*, 2000).

1.4.2 (a) Humoral immunity

When triggered by cognate antigen, B cells (in the presence of T cell helper activity) are activated and differentiate into plasma cells which secrete antibodies that can act in various ways to combat extracellular pathogens and may also target infected cells. Although cell-mediated immunity is typically more important in elimination of established virus infections, humoral immunity can play an important role by limiting the spread of infection and thereby contributing to containment of viral replication (reviewed in (Casadevall & Pirofski, 2003)). In addition, pre-existing antibodies play an important role in preventing re-infection on secondary exposure to a particular pathogen.

Neutralising antibodies can bind directly to viruses and may limit their infectivity by interfering with their interaction with host cell receptors or blocking infection at post-entry steps, and thus prevent viruses from establishing primary infection. Antibodies can also interact with components of the innate immune response to aid destruction of pathogens. Antibodies can bind to pathogens and opsonise them for recognition by cells which express Fc receptors that are able to bind to the constant region of the antibody, for example phagocytic cells or NK cells (the latter promoting killing by ADCC). Alternatively, antibodies binding to the surface of a pathogen can activate complement via the classical pathway. Complement-coated pathogens can then be bound by CR-bearing phagocytic cells or destroyed by the terminal MAC complex assembled upon activation of complement.

1.4.2 (b) Cell-mediated immunity

The activities of virus-specific $CD4^+$ and $CD8^+$ T cells make up the antiviral cell-mediated immune response. $CD4^+$ T cells predominantly provide helper functions, not only for virus-specific $CD8^+$ T cell responses, but also for antibody responses. $CD8^+$ T cells on the other hand are the cell type which is mainly responsible for the direct cytotoxic and other antiviral activities of the cell-mediated immune response.

- The $CD4^+$ T cell response

CD4 T cells are activated upon recognition of peptides presented by MHC class II molecules on antigen presenting cells. Peptide antigens 10-12 amino acids in length are generated from extracellular antigens taken up by endocytosis. The acidification of endocytic vesicles activates proteases which degrade the antigens into peptide fragments. MHC class II molecules are

synthesised in the ER, and their peptide binding groove occupied by the invariant chain to prevent peptide binding. The invariant chain directs newly synthesised MHC class II molecules to vesicles which fuse with endocytic vesicles containing peptide fragments. The invariant chain is released to allow peptides to bind, a process assisted by HLA-DM, and MHC class II: peptide complexes are assembled and transported to the cell surface for recognition by $CD4^+$ T cells.

Via the provision of cytokines and via cell-cell contact mediated mechanisms, $CD4^+$ T cells play an important role in orchestrating both the antibody response and the $CD8^+$ T cell response to an invading pathogen. $CD4^+$ T cells provide important activation and co-stimulatory signals to B cells for the production of antibodies, and also for isotype class switching. $CD4^+$ T cell-deficient mice are unable to generate strong neutralising antibody (nAb) responses. $CD4^+$ cells also have important roles in the priming of CD8 responses (Ridge *et al.*, 1998), maintenance of $CD8^+$ T cell memory (Battegay *et al.*, 1994) and maturation of $CD8^+$ T cell function (Zajac *et al.*, 1998).

The requirement for CD4 help during the induction of a CD8 response is controversial and seems dependent on the nature of the antigen. While earlier studies focused on the secretion of cytokines such as IL-2 as being the key role played by $CD4^+$ cells in activation of $CD8^+$ T cells (Keene & Forman, 1982), later studies focused on the role of $CD4^+$ T cells in APC activation (which in turn facilitates the induction of antigen-specific CD8 responses). $CD4^+$ cells provide co-stimulatory signals for the activation of APCs via CD40L-CD40 signalling (Bennett *et al.*, 1998; Ridge *et al.*, 1998; Schoenberger *et al.*, 1998) (and possibly other membrane-membrane receptor ligand interactions such as TNF-related activation induced cytokine (TRANCE) and TRANCE receptor (Bachmann *et al.*, 1999)), resulting in effective priming of a CD8 response. During some virus infections, sufficient levels of APC activation are induced by the virus and/or associated innate immune response to obviate the requirement for CD4 help (for example during LCMV infection of mice (Christensen *et al.*, 1994; Leist *et al.*, 1987; Moskophidis *et al.*, 1987a)). A number of studies have however provided evidence for a more critical requirement for CD4 help in memory cell generation. In murine infection models, depletion of CD4 cells during the priming phase was found not to have a major effect on the primary response mounted, but resulted in impaired responses by memory cells to reinfection (Janssen *et al.*, 2003; Shedlock & Shen, 2003; Sun & Bevan, 2003).

The importance of CD4-CD8 cell collaboration in controlling persistent viral infections is highlighted by experiments carried out in CD4 deficient mice infected with LCMV. In the absence of CD4 help, infection with a high dose of virus or rapidly replicating or disseminating strains of LCMV led to loss of virus-specific CTLs and establishment of viral persistence (Battegay *et al.*, 1994; Matloubian *et al.*, 1994). Furthermore, in a model of chronic LCMV infection it was revealed the CD8 response may be impaired in the absence of helper function by either the deletion of CTL, or the functional silencing of CTL (Zajac *et al.*, 1998).

- The $CD8^+$ T cell response

$CD8^+$ T cells recognise peptide antigens on the surface of APCs bound to MHC class I molecules. Peptide fragments 8-10 amino acids in length with appropriate termini for binding to MHC class I molecules are generated by degradation of cytosolic proteins by the proteasome, a large mutlicatalytic protease complex. The peptides are transported into the endoplasmic reticulum by the transporter associated with antigen processing (TAP) and are assembled together with newly-synthesised α chain:$\beta_2$microglobulin (β_2m) complexes to form MHC-peptide complexes which are transported to the cell surface via the Golgi apparatus for presentation to $CD8^+$ T cells. In addition to endogenously processed peptides, dendritic cells are able to present exogenously acquired peptides (cross-presentation).

In response to signalling through the T cell receptor, CD8 cells are triggered to undergo clonal expansion and differentiation into functional effector cells, CTL which can lyse infected cells. CTL can induce apoptosis of target cells using two distinct mechanisms: a calcium-dependent granule exocytosis pathway involving the release of perforin and granzymes from preformed cytolytic granules; and a calcium-independent pathway involving the ligation of death receptors on susceptible cell types by death-inducing ligands such as Fas L and TNF (reviewed in (Barry & Bleackley, 2002; Catalfamo & Henkart, 2003; Thorburn, 2004)). Virus-specific $CD8^+$ T cells can also produce soluble factors which mediate anti-viral effects, such as IFN-γ and TNF-α.

By virtue of their functions, $CD8^+$ T cells play a key role in the immune response to many pathogens (reviewed in (Harty *et al.*, 2000; Wong & Pamer, 2003)). For example, $CD8^+$ T cells are essential for virus clearance in acute LCMV infection (Fung-Leung *et al.*, 1991; Gegin & Lehmann-Grube, 1992), and chronic infections such as Epstein Barr virus (EBV), CMV and HTLV-1 are

perpetually held in check by CD8 T cells (Bieganowska *et al.*, 1999; Moss & Khan, 2004; Rickinson & Moss, 1997). The relative importance of 'curative' and lytic mechanisms of $CD8^+$ T cell control of viral infection may be dependent on the virus, the stage of infection and host cell type (Guidotti & Chisari, 2001). For example, it has been shown using perforin-deficient mice that in acute LCMV infection perforin-mediated destruction of infected cells is critical for viral clearance (Kagi *et al.*, 1994; Walsh *et al.*, 1994). However, in an infection such as hepatitis B, viral clearance may involve non-cytopathic cytokine-dependent 'curative' processes. Using a transgenic mouse model of hepatitis B virus (HBV) infection, it was shown that CTLs producing IFN-γ and TNF-α were able to inhibit viral gene expression and replication without killing the hepatocyte (Guidotti *et al.*, 1996; Guidotti *et al.*, 1994).

1.4.3 Utility of murine virus infection models for elucidation of mechanisms involved in antiviral immunity

Murine virus infection models can provide several advantages in the elucidation of general concepts in immunity and antiviral defence. The possibility of studying infection with well-defined viruses within inbred hosts, sampling multiple timepoints and/or tissues, their amenity to experimental manipulation and the availability of reagents such as transgenic and knockout mice can prove to be invaluable for such purposes. Two of the murine virus infection models that have been most extensively utilised in the field of viral immunology are the LCMV and influenza virus infection models.

LCMV is an arenavirus which is a natural pathogen of mice. The outcome of infection with LCMV is dependent on both viral (e.g. virus isolate, dose and route of administration) and host factors (age, genetic background and immunocompetence) (reviewed in (Borrow and Oldstone, 1997)). Infection of adult immunocompetent mice by the intraperitoneal or intravenous route with moderate doses of most LCMV isolates results in an acute infection which is cleared within 7-10 days. $CD8^+$ T cells play a key role in mediating virus clearance (Fung-Leung et al., 1991; Moskophidis et al., 1987; Murali-Krishna et al., 1998) although optimal control of virus replication also requires input from other arms of the immune response (Klenerman, 2004). This infection system therefore provides a good model for studying mechanisms involved in acute control of systemic virus infection.

By contrast, infection of mice in utero or neonatally, or infection as adults with high doses of certain LCMV isolates results in the establishment of persistent infection. This provides a good model for studying mechanisms underlying the failure of immune control of virus infection and defining therapeutic approaches for combating viral infections.

Influenza viruses belong to the Orthomyxoviridae family of viruses. A number of genera exist. Influenza viruses can naturally occur in many species, but mice can be experimentally infected with influenza A or B viruses to study aspects of pathogenesis and immune responses to the virus. Productive infection and high titre viral replication is limited to the respiratory tract (Rott et al., 1995; Walker et al., 1992). Respiratory infection of $H2^b$ mice causes an acute pneumonia, with the virus being cleared by day 10 after infection (Allan et al., 1990). This model thus allows for the study of a localized, transient infection (Turner et al., 2004; Woodland and Randall, 2004).
Mice that have been infected with a particular strain of influenza are substantially protected for life, providing a good system for studying memory immune responses (Woodland et al., 2001) and cross-protection against infection by different viral subtypes (Tamura et al., 2005). Although neutralising antibody responses are critical for protection from influenza virus infection (Gerhard, 2001), various components of the immune response act together to provide optimal protection (Doherty et al., 1997; Woodland et al., 2001).

The value of using the LCMV and influenza virus infection models is evidenced by the many important concepts which have been defined using these models. These include the demonstration of MHC restriction of $CD8^+$ T cell responses (using the LCMV model (Zinkernagel and Doherty, 1974a; Zinkernagel and Doherty, 1974b)); the roles of different arms of the immune response in protection and prevention of re-infection (both LCMV (Khanolkar et al., 2002; Klenerman, 2004) and influenza (Tamura and Kurata, 2004)); the concept that antiviral immunity can contribute to disease pathogenesis (as in the case of meningitis associated with LCMV infection (McGavern et al., 2002) and pneumonia caused by influenza virus infection (Tsurita et al., 2001)); definition of mechanisms involved in the induction of immune responses to infections acquired by a mucosal route (influenza (Brandtzaeg, 2003; Tamura and Kurata, 2004)); and characterisation of the mechanisms contributing to

viral persistence (LCMV) such as neonatal tolerance (Pircher et al., 1989), CTL exhaustion (Moskophidis et al., 1993) and CTL escape (Pircher et al., 1990).

In this thesis I also made use of the well characterised LCMV and influenza virus murine infection models to address the effects of limited $CD4^+$ T cell help at the time of immune priming on the antiviral $CD8^+$ T cell responses induced under such conditions. More specifically, I explored whether a lack of CD4 help results in biasing of the response to epitopes (with their resultant immunodominance) that are targeted by $CD8^+$ T cells with less stringent activation requirements, e.g. high affinity epitopes or epitopes that are able to be cross-recognised by memory T cells.

1.5 Viral immune evasion strategies

Viruses have evolved the means to co-exist in their human hosts by employing various strategies to evade elimination by host immune responses. Various viral-encoded factors mediating immune evasion may play a role in virulence, as evidenced by the attenuation of viruses carrying targeted mutations of such factors (Fleming *et al.*, 1999; Saederup *et al.*, 1999; Tortorella *et al.*, 2000). The large genome of certain DNA viruses, such as pox viruses and herpes viruses, allows them to devote a large number of genes to immune defence; many are viral homologues of host genes (Alcami & Koszinowski, 2000). RNA viruses, which typically have a more limited genome size are not as able devote individual genes to host control, and therefore proteins encoded by RNA viruses are multifunctional, and they frequently employ rapid replication and mutation as means to evade immune control (Lucas *et al.*, 2001).

The mechanisms used by viruses to subvert the host immune response can be broadly divided up into three categories: those that allow viruses to avoid recognition by the antiviral immune response, those that impair the response, and those that confer resistance to immune effector mechanisms. Each of these are reviewed in the following section, with those specific immune evasion strategies used by HIV-1 discussed in section 1.7.

1.5.1 Strategies to avoid recognition by the antiviral immune response

(a) Latency

Some viruses avoid the host immune response by downregulating the expression of viral gene products during certain phases of the replication cycle

in a state which is known as latency. With minimal expression of viral proteins and therefore few virus-derived peptides available for presentation on the cell surface, virus-infected cells are essentially invisible to immune surveillance. Such latent infections can be reactivated periodically, leading to recurrent illness. This is a period in which the virus undergoes productive replication and infection to allow transmission to new hosts. Examples of viruses which can enter latency are HSV (latent in sensory neurons), VZV (latent in dorsal root ganglia) and EBV (latent in B cells). Primary infection with EBV is controlled by virus-specific $CD8^+$ T cells (reviewed in (Rickinson & Moss, 1997)). However EBV remains quiescent in a fraction of B cells. These latently infected cells express few viral proteins. One protein which is needed to maintain the viral genome, Epstein Barr virus nuclear antigen (EBNA) -1, is expressed but for reasons explained later EBNA-1 is unable to elicit a T cell response.

(b) Infection of immune privileged sites

By infecting areas with little immune surveillance, viruses are able to hide from the immune system. Such areas include the CNS, the kidney, salivary glands and the anterior chamber of the eye. Viruses which can infect the CNS (such as the JC polyoma virus, varicella zoster virus and measles virus) are protected by the blood brain barrier, which limits trafficking of immune cells, low levels of MHC expression on cells of the CNS, and a generally immunosuppressive environment.

(c) Interference with MHC class I antigen presentation

Virus-derived peptides must be presented on the cell surface in the context of MHC class I in order to be recognised by antigen specific $CD8^+$ T cells. Antigen presentation can be prevented at several different stages to enable the virus to escape recognition by the CD8 response. The generation of peptide, transport of the antigenic peptide into the ER via TAP and surface expression of MHC class I-bound peptide are all subject to interference by viruses.

That inefficient antigen processing results in failure of CD8 recognition is evidenced by the EBNA-1 protein of EBV. The failure of EBNA-1-specific CTL to be activated is attributed to the resistance of EBNA-1 to proteolysis due to a long stretch of gly-ala repeats in its sequence (Levitskaya *et al.*, 1995). This sequence is not included in EBNA -2, -3 and -4, which are degraded,

presented and do stimulate CTL responses (Khanna *et al.*, 1992; Murray *et al.*, 1990).

Certain viral gene products (for example HSV ICP47 (Fruh *et al.*, 1995; Hill *et al.*, 1995; York *et al.*, 1994) and human cytomegalovirus (HCMV) US6 (Ahn *et al.*, 1997; Hengel *et al.*, 1996; Hengel *et al.*, 1997; Lehner *et al.*, 1997)) are able to inhibit peptide transport from the cytosol into the ER where MHC class I-peptide complexes are assembled by interfering with TAP function, e.g. HCMV US6 prevents peptide transport possibly by occluding the exit pore of the TAP complex. Consequently, peptide loading of class I molecules is unable to occur.

Various mechanisms are used by different viruses to target the maturation, assembly and export of MHC class I molecules. HCMV gpUS2 and gpUS11 mediate the downregulation of MHC class I molecules on the cell surface by selectively targeting class I heavy chains for degradation by the proteasome (Wiertz *et al.*, 1996a; Wiertz *et al.*, 1996b), whereas the product of gpUS3 retains class I molecules in the ER (Ahn *et al.*, 1996; Jones *et al.*, 1996), as does the E3/19K product of adenovirus (Cox *et al.*, 1991). Murine cytomegalovirus (MCMV) m152, through a retention signal, retains class I molecules within the ER-Golgi intermediate compartment, preventing surface expression (Ziegler *et al.*, 1997).

Kaposi's sarcoma-related herpes virus/HHV type-8 (KSHV/HHV-8) K3 and K5 proteins enhance the endocytosis of MHC class I from the cell surface, although with varying degrees of specificity for different HLA alleles (Coscoy & Ganem, 2000; Ishido *et al.*, 2000). K3 mediates enhanced internalisation of HLA-A, -B, -C and -E whereas K5 is implicated in rapid endocytosis of HLA-A and -B, and a lesser amount of endocytosis of HLA-C. The MCMV m06 gene product binds MHC class I molecules and targets them to the lysososme for degradation (Reusch *et al.*, 1999).

(d) Avoiding recognition by NK cells

The downregulation of MHC class I on the cell surface, used as a mechanism to avoid $CD8^+$ T cell recognition, is associated with the increased susceptibility of such cells to attack by NK cells since certain MHC molecules act as ligands for inhibitory receptors on NK cells.

This may be avoided by allele-specific MHC class I downregulation. HLA-A, -B and -C locus products function primarily in presentation of peptide to CTL, whereas HLA -E and -G locus products may function primarily in inhibition of

NK cell-mediated lysis. HCMV infection of cells results in the partial downregulation of MHC class I, sparing some HLA-C alleles, thereby providing a ligand for the inhibitory receptor KIR2D (Huard & Fruh, 2000; Schust *et al.*, 1998; Valiante *et al.*, 1997). In addition, HCMV is able to upregulate the expression of HLA-E (Tomasec *et al.*, 2000; Ulbrecht *et al.*, 2000), a nonclassical MHC class I molecule that interacts with the NK cell inhibitory receptor CD94/NKG2A, B and C to prevent lysis by NK cells (Borrego *et al.*, 1998; Braud *et al.*, 1998).

Another mechanism to avoid the NK response is via the production of class I homologues that may be able to substitute for the inhibitory MHC class I ligands and prevent NK cell activation, for example the class I homologue, UL18, carried by HCMV (Beck & Barrell, 1988).

(e) Interference with MHC class II antigen presentation

Preventing Class II antigen presentation is a way of interfering with activation of helper T cells (which may impact on other responses dependent on T cell help). As for the inhibition of MHC class I antigen presentation, the processing of antigenic peptides or MHC class II expression can be interfered with by viruses to prevent presentation of viral antigens by MHC class II to $CD4^+$ T cells.

Virus encoded proteins may affect processing of proteins in the endocytic pathway. Human- and bovine- papillomavirus (HPV and BPV) encode E5 and E6, proteins which interact with components of the endocytic pathway. E5 (encoded by both) interferes with a proton pump required for endosomal acidification (Andresson *et al.*, 1995) and may disrupt processing of antigenic proteins by altering events that take place in the endocytic pathway. E6 produced by BPV binds to AP1 (Tong *et al.*, 1998), an adaptor protein associated with clathrin-coated vesicles in the trans-Golgi network, and also may affect processing of proteins.

The inhibition of surface expression of class II molecules can be mediated by both transcriptional and post-translational effects. Adenovirus, MCMV and HCMV are all able to affect class II transcription. HMCV US2 translocates the MHC class II DRα and DMα chain into the cytosol for degradation by the proteasome (Tomazin *et al.*, 1999).

Viruses can also indirectly inhibit the expression of MHC class II by interfering with IFN-induced expression of MHC class II, for example MCMV (Heise & Virgin, 1995) can interfere with IFN-signalling pathways.

(f) Antigenic variation

Pathogens can also evade immune surveillance by altering their antigenic composition. Antigenic variation can be achieved by several different mechanisms including reassortment (for viruses with a segmented genome), recombination and mutation. Mutation is common for RNA viruses which have an RNA polymerase that lacks proof reading ability. Their high mutation rate and short replication times enable them to rapidly adapt in response to environmental changes such as immune selection pressure. Thus the generation and selection of variants which can evade recognition by neutralising antibodies is common in RNA viruses, for example equine infectious anaemia virus (Montelaro *et al.*, 1984; Salinovich *et al.*, 1986) and visna virus (Scott *et al.*, 1979). In influenza virus infection, the emergence of point mutations that alter the binding sites for protective antibodies on the virion surface allows the virus to evade neutralisation by antibodies. Here the mutation allows for reinfection of a host rather than promoting persistence. Mutation in viral CTL epitopes can also abrogate epitope-specific $CD8^+$ T cell responses by modifying peptide processing or transport, altering peptide binding to MHC class I molecules or peptide interaction with the TCR. This is discussed in more detail in the context of HIV-1 infection in section 1.7.

1.5.2 Strategies which impair the host antiviral immune response

Viruses can impair the immune response in order to establish persistent infections within a host. In the case of some viruses, infection can be associated with a generalised immunosuppression; in many virus infections, more restricted defects in the virus-specific immune response are associated with virus persistence.

(a) Generalised immunosuppression

Measles is a leading cause of childhood disease and is associated with a transient profound immunosuppression. Infection can be fatal, the cause of death usually being a secondary bacterial infection, such as pneumonia, caused by measles-induced immunosuppression.

Measles virus can infect T cells, B cells, monocytes and DCs *in vitro* and induce syncytium formation or apoptosis and thereby cause the destruction of key immune cells (Joseph *et al.*, 1975). Besides causing the loss of cells, other immune responses are impaired due to reduced T and B cell function (reviewed in (Borrow & Oldstone, 1995; Klagge & Schneider-Schaulies,

1999)), such as reduced delayed type hypersensitivity (DTH) responses (children have increased susceptibility to mycobacterial infections) and impaired antibody (Casali *et al.*, 1984; McChesney *et al.*, 1986; Tishon *et al.*, 1996) and IL-12 (required for activation of Th1 $CD4^+$ T cells) production (Fugier-Vivier *et al.*, 1997; Karp *et al.*, 1996). Two structural components of the measles virus may contribute to measles-induced immunosuppression. The virus nucleocapsid protein binds to FcγRII and delivers negative signals to attenuate B cell antibody production *in vitro* (Ravanel *et al.*, 1997); and measles haemagglutinin binding to CD46 suppresses IL-12 production by macrophages (Karp, 1999; Karp *et al.*, 1996).

(b) Impairment of cytokine production

IFNs are an important class of cytokines which both mediate immunoregulatory activities and have a key role in protecting cells from viral infection. Viruses are able to impair IFN induction by blocking the synthesis or activity of factors required for their production. For example IL-18 and IL-12 are required for the production of IFN-γ. CrmA from Cow pox virus inhibits caspase-1 (also known as IL-1β converting enzyme, or ICE), which in turn may inhibit the maturation of IL-18 (Smith *et al.*, 1998; Smith *et al.*, 1997); various poxviruses encode soluble IL-18 binding proteins (Smith *et al.*, 2000; Xiang & Moss, 1999); measles virus binds CD46 in macrophages and inhibits IL-12 production (Karp, 1999); and herpes and poxviruses express IL-10 homologs that diminish the Th1 response by downregulating production of IL-12 (Kotenko *et al.*, 2000; Spriggs, 1996). Impaired IFN induction could impede local control of virus replication and potentially have consequences for activation of the innate and/or adaptive immune response.

As with the IFNs, the activity of other cytokines can be also modulated or inhibited through interference with their production. HCMV inhibits the transcription of the chemokine monocyte chemoattractant protein-1 and, as already mentioned, measles virus inhibits production of IL-12 by monocytes, macrophages and DCs (Karp, 1999; Karp *et al.*, 1996). Viruses which encode homologues of IkB are able to block cytokine expression mediated by NFκB and the nuclear factor of activated T cells (NFAT) transcription factors (e.g. African swine fever virus (Miskin *et al.*, 1998)).

The process of cytokine maturation may also be interrupted, e.g. pox viruses synthesise inhibitors of ICE (Kettle *et al.*, 1997; Ray *et al.*, 1992), preventing

secretion of mature IL-1β, and reducing the inflammatory response triggered by this cytokine.

(c) Neonatal tolerance

Some viruses are able to establish persistent infections if they infect the host early enough during life at the time when tolerance is being established. During T cell maturation in the thymus in early life, clones that bind with high affinity to antigens presented in the thymus are eliminated by negative selection as a mechanism to delete cells that have the potential to react with self antigens. Hence, for example, if mice are infected with LCMV *in utero* or as neonates, viral antigens will be present in the thymus to cause deletion of LCMV-specific T cells as they begin to mature. The peripheral $CD8^+$ T cell repertoire that develops will be unable to clear the infection and the virus will be able to persist (Pircher *et al.*, 1989). The clonal deletion of HBV-specific T cells also occurs following transplacental HBV infection. A reduction in the frequency of HBV-specific T cells may also account for the increased likelihood of developing chronic hepatitis if an individual is infected neonatally compared to during adulthood (Chisari & Ferrari, 1997).

(d) Immune Exhaustion

Immune exhaustion is another way of enabling a persistent infection to be established. T cell exhaustion, defined here as the rapid disappearance of antigen-specific T cells, is likely to occur in the face of a high antigen load, as may be generated with a virus which replicates to high titres and spreads rapidly in lymphoid tissues. This has been shown for more aggressive strains of LCMV such as LCMV DOCILE (Moskophidis *et al.*, 1993). If stimulated under conditions of antigen excess, T cells may undergo apoptosis rather than the usual response of expansion and differentiation. The mechanisms behind this are not fully understood, but may involve the incorrect priming of T cells by non-professional APCs presenting viral peptides or by professional APCs whose functions have been impaired, resulting in T cell apoptosis; or CD8 cells becoming a target for LCMV-specific CTL as a result of their direct infection (Borrow *et al.*, 1991) or the acquisition of peptide-MHC complexes from APC (Huang *et al.*, 1999).

(e) Induction of T cells with impaired effector functions

Another way in which viruses can evade clearance by T cell-mediated immunity is by inducing a T cell response which is functionally impaired. Primary hepatitis C virus (HCV) infection has been associated with transient dysfunction of antigen-specific T cells, where temporarily 'stunned' cells are unable to produce IFN-γ and do not proliferate *in vitro* (Lechner *et al.*, 2000a; Thimme *et al.*, 2001). In addition, the perforin content and lytic activity of virus-specific T cells have been observed to be significantly impaired during primary HCV infection (Urbani *et al.*, 2002). This impairment is fully reversible when viraemia falls and HCV-specific CD4 responses become detectable but persists when chronic infection develops, and so the defective function of HCV-specific CD8 cells may contribute to viral persistence in chronic infection. That function is restored when the viral load falls suggests that the dysfunctional phenotype may have been induced by the high antigen levels during acute infection. Overstimulation of cells may induce anergy or be associated with presentation of viral antigens by non-professional APCs which may aberrantly activate T cells.

However, the impaired effector function of HCV-specific $CD8^+$ T cells observed during chronic infection is associated with weak HCV-specific CD4 responses (Wedemeyer *et al.*, 2002), giving rise to the possibility that the virus-specific $CD8^+$ T cells may be arrested in an immature state due continuous antigenic stimulation in the absence of sufficient CD4 help. Furthermore, impaired effector function is associated with incomplete effector cell differentiation (Wedemeyer *et al.*, 2002), and so HCV may block or divert the differentiation of HCV-specific cells.

That conditions of helper cell deficiency may contribute to the functional impairment of cells is supported by observations made in a chronic LCMV model (Zajac *et al.*, 1998). LCMV-specific $CD8^+$ T cells specific for a dominant epitope persisted but were noncytotoxic and did not produce IFN-γ, contributing to the inability to clear infection. This functional unresponsiveness was more marked under conditions of CD4 deficiency. What may happen during HIV infection, which is associated with a deficit in $CD4^+$ T cell frequency and function, is the subject of discussion later on.

1.5.3 Strategies which aid viral resistance to immune effector mechanisms

(a) Resistance to the effects of cytokines and chemokines

Another strategy used by viruses to evade the host immune response involves interference with the effector functions of cytokines induced by viral infection. Cytokines and chemokines coordinate a variety of events in the immune response, including inflammation, cellular activation, proliferation, differentiation and chemotaxis. By acting as immune mediators, they orchestrate the induction and maintenance of innate and adaptive antiviral responses. A number of cytokines and some chemokines also have direct antiviral effects. By interfering with their actions, viruses are able to avoid such cytokine-mediated activities.

- IFNs

As already mentioned, IFNs are a family of cytokines which have important antiviral activity and have a role in the activation of cells within the innate and adaptive immune systems. Upon binding of IFNs to their receptors, a signal transduction cascade is initiated that leads to activation of IFN-inducible genes (reviewed in (Schindler, 1999)). Following receptor binding, tyrosine phosphorylation of the receptor-associated tyrosine kinases (Janus kinase (Jak) 1 /Tyk2 for type I IFNs; Jak1/Jak2 – for type II IFNs), as well as tyrosine phosphorylation of the individual receptors, takes place. Once activated, the Janus kinases phosphorylate a family of proteins, the STAT (signal transducers and activators of transcription) proteins. This leads to dimerisation of different STATs and their translocation into the nucleus. There, they assemble with other factors to become large transcriptional activation complexes. These include genes which switch the responding cell into an 'anti-viral state' which culminates in the arrest of protein translation and cellular proliferation, the upregulation of enzymes that degrade viral RNA and apoptosis. One protein induced is an enzyme (2' 5' oligoadenylate synthetase (2' 5' OAS)) which activates an endoribonuclease (RNase L) that degrades viral RNA. IFN also induces the synthesis of ds RNA-dependent protein kinase (PKR). This phosphorylates eIF-2, an initiation factor for protein synthesis, thereby inhibiting translation. Many viruses are able to interfere with the activities of IFNs by targeting various points along the signalling pathway (reviewed in (Cebulla *et al.*, 1999)).

Poxviruses encode soluble versions of receptors for IFNs (which may be localised at the cell surface) that block the functions of IFNs (Smith *et al.*,

1998). For example vaccinia virus (VV) secretes a type I IFN receptor homolog B18-R (Symons *et al.*, 1995) and a type II receptor homolog B8-R (Alcami & Smith, 1995) which can bind to and inhibit IFN-α and -γ respectively. Viruses can use various methods to inhibit the JAK/STAT pathway to abrogate IFN signalling. Many viruses (Simian virus 5, Sendai virus (Didcock *et al.*, 1999b), human parainfluenza viruses (Young *et al.*, 2000), adenovirus (Leonard & Sen, 1996)) have effects on STATs to block either or both type I or II responses. For example, the V protein of Simian virus 5 causes proteasome-mediated degradation of STAT 1, needed for both type I and II IFN signalling (Didcock *et al.*, 1999a). HMCV diminishes levels of Janus kinase-1, which interferes with IFN-α-stimulated gene expression (Miller *et al.*, 1998).

Interferon regulatory factors (IRFs) are proteins that regulate IFN-induced transcription, and can be manipulated by viruses to inhibit transcriptional responses induced by viruses. HHV-8, for example, encodes proteins with homology to IRFs which may modulate the expression of IFN-inducible genes (Burysek *et al.*, 1999; Li *et al.*, 1998a).

Viruses use various strategies to inhibit the activity of PKR, an RNA-dependent enzyme that is part of an IFN-induced mechanism to inhibit protein synthesis. Adenovirus and EBV encode RNAs that inhibit PKR activation (Clemens, 1993; Ma & Matthews, 1996; Sharp *et al.*, 1999); HCV E2 binds to and inhibits PKR activation (Taylor *et al.*, 1999); and HSV-1 counteracts PKR activity by activating a phosphatase to dephosphorylate eIF2-α (He *et al.*, 1997).

The RNase L system, which degrades viral RNAs, is also targeted by viruses, for example HSV, which produces antagonists of 2' 5' OAS, the enzyme responsible for activating RNase L (Cayley *et al.*, 1984).

- Other cytokines/chemokines

Viruses are able to produce homologues of a range of cytokines and cytokine receptors. Mimicry of cytokines might redirect the immune response for the benefit of virus, for example they might use the homologue to induce signalling in the infected cell in ways which might promote virus replication. Members of the herpes family viruses produce vIL-6 and -17, which might have immunomodulatory activity but might also increase proliferation of cells that are targets for viral replication (Spriggs, 1996). As already mentioned for the

IFN receptor homologues, cytokine receptors (or cytokine binding proteins) may be able to neutralise cytokine activity.

Virus-encoded chemokines are either antagonists that block leukocyte recruitment to sites of infection, or agonists that enhance recruitment of cells for their advantage, for example to support viral replication or prevent Th1 anti-viral responses. KSHV synthesises vMIP-II, a broad-chemokine receptor antagonist, which blocks monocyte chemotactic responses to RANTES (Regulated on activation, normal T cell expressed and secreted), MIP-1α and MIP-1β (Kledal *et al.*, 1997). The HHV-6 chemokine U83 binds to chemokine receptors and may recruit mononuclear cells to sites of viral replication, facilitating virus dissemination (Zou *et al.*, 1999). HHV-8 produces the chemokine vMIP-1 which interacts with CCR8, a chemokine receptor preferentially expressed by Th2 cells. Thus HHV-8 may use vMIP-1 to selectively recruit and activate Th2 cells to areas of viral infection, biasing the Th1/2 balance of the antiviral immune response (Dairaghi *et al.*, 1999; Endres *et al.*, 1999).

Another way in which the activities of cytokines/chemokines can be influenced is by alteration of their interaction with host receptors. Viruses may encode secreted receptors which compete with cellular receptors for cytokine/chemokine binding. Alternatively, they can use membrane-bound receptors to interfere with target cell recognition of cytokines.

Many poxviruses are able to produce TNF receptor homologues to interfere with the interaction of TNF with cellular receptors (Alcami *et al.*, 1999; Loparev *et al.*, 1998), thereby inhibiting the antiviral effects of TNF, including induction of apoptosis (discussed later).

Binding proteins for chemokines may also provide a way of neutralising their activity, for example MCMV encodes several secreted glycoproteins with homology to the IL-18 binding protein (Smith *et al.*, 2000; Xiang & Moss, 1999). These are able to sequester IL-18, which is involved in NK cell activation and induction of Th1 responses.

Membrane-bound cytokine receptors can often retain their signalling function (such as the HCMV chemokine receptor homolog US28 (Billstrom *et al.*, 1998)), and alter the cell physiology in response to cytokine signals. Such signals may force the cells to create an environment that is of direct benefit to the virus, for example by allowing more efficient replication or by blocking apoptosis.

Viruses are also able to inactivate host cytokine receptors directly. For example, HHV-6 and -7 downregulate the expression of CXCR4 upon infection of $CD4^+$ T cells (Yasukawa *et al.*, 1999), resulting in diminished calcium flux and chemotaxis in response to CXCR4 ligand.

(b) Resistance to antibody and complement-mediated immunity

Viruses can also evade the effector activities of responses involving antibodies and complement.

Some viruses encode viral Fc receptors (FcRs). The binding of non-immune IgG to these receptors may sterically restrict the access of virus-specific immune IgG. The FcRs can also bind to exposed Fc domains of antiviral antibodies after the Fab region binds to the virus antigen and prevent subsequent Fc-dependent immune activation of complement and phagocytes. The HSV-1 gE and gI glycoproteins form a complex that binds IgG on HSV-1 virions and on infected cells, and so gE/gI function may account for the relative ineffectiveness of anti-HSV Ig against HSV infection (Baucke & Spear, 1979; Johnson *et al.*, 1988).

In order to limit complement activation and neutralisation of virus particles, some viruses possess homologues of complement control proteins or can utilise host control proteins that act at various stages along the pathway of complement activation.

Poxviruses, herpesviruses and retroviruses are able to encode proteins with functional similarities to complement control proteins which inhibit the complement cascade (reviewed in (Favoreel *et al.*, 2003)), for example herpes virus saimiri encodes a CD59 homolog that inhibits C9 polymerisation and formation of the terminal membrane attack complex pore (Rother *et al.*, 1994).

Some viruses alter the cellular expression of host complement control proteins, for example, HCMV upregulates CD46 and CD55 expression on infected cells *in vitro* (Spiller *et al.*, 1996).

Also, viruses that bud through the plasma membrane can acquire host membrane proteins, including regulators of complement activation such as CD46, CD55 and CD59, and incorporate them into the viral envelope (e.g. vaccinia virus (Vanderplasschen *et al.*, 1998)).

(c) Resistance to apoptosis

Viruses depend on living cells for replication, but viral infection often triggers apoptosis of infected cells. This can occur as a direct effect of the virus

infection, or the cell simply self-destructing after sensing some unanticipated occurrence that affects normal cellular homeostasis, such as unscheduled DNA synthesis; or alternatively may be triggered by immune effector pathways. Prolonging the survival of infected cells is clearly in the interests of the virus, to allow it to complete its full replicative cycle, mature and disseminate. It is therefore not surprising that many viruses encode proteins that inhibit apoptosis.

Pathways for the induction of apoptotic death contain molecules that can serve as targets for viral-encoded proteins. These pathways involve:

- Death receptors (DRs) on the cell surface (e.g. TNF-R1, Fas, DR4 and DR5), which interact with specific ligands (TNF-α, FasL and TNF-related apoptosis-inducing ligand (TRAIL)). Binding to their ligands causes the oligomerisation of receptors which can transduce death signals through death domains.
- The participation of mitochondria and the release of cytochrome c into the cell cytosol. This is regulated by members of the Bcl-2 family which form heterodimers, including antiapoptotic molecules bcl-2 and bcl-XL, and death-promoting molecules, bad, bax, or bak.
- The release of perforin and granzymes from the cytoplasmic granules of cytolytic cells.

Most apoptotic stimuli, regardless of which pathway they trigger, converge on and activate, a common set of caspases. Caspases comprise a family of aspartate-specific cysteine proteases that orchestrate the apoptotic program by proteolytically activating other caspases and other cellular targets, leading to DNA fragmentation and cell death.

The apoptotic pathway can be targeted at several points by viral anti-apoptotic mechanisms. The modulation of death-inducing ligands or their receptors can be one level at which viruses can prevent apoptosis. The adenovirus E3-10.4/14.5K proteins cause the internalisation of Fas from the cell surface and its degradation inside lysosomes (Tollefson *et al.*, 1998). Also, various viruses encode TNF receptor homologues which can bind and block the action of the death-inducing cytokine (e.g. rabbit myxoma virus, M-T2 (Schreiber *et al.*, 1997)).

Following the ligation of death receptors, death signals are relayed through adaptor molecules, Fas-associated and TNF receptor-associated death domains (FADDs and TRADDs), to upstream, initiator caspases (e.g. caspase

-8 (FLICE) and -10). A caspase cascade ensues, with downstream caspases (caspase -3, -6 and -7) being active during the more terminal stages of death. The FADD-caspase contacts rely on interactions between death effector domains in each. To interfere with this interaction, several viruses encode death-effector-domain-containing proteins called viral FLICE inhibitory proteins (vFLIPs). These bind FADD and/or caspases and prevent recruitment and activation of initiator caspases upon ligation of death-inducing ligands. vFLIPs are encoded by several herpes viruses. For example, KSHV/HHV-8 ORF71 disrupts the recruitment of caspase 8 to FADD (Thorne *et al.*, 1997).

Inhibition of caspase activity is another way in which viruses can prevent apoptosis in response to apoptotic stimuli. crm A encoded by cowpox virus can block the activity of caspase 8 and caspase-1 (Dbaibo & Hannun, 1998). Other viruses which encode caspase inhibitors include adenovirus (14.7K) (Chen *et al.*, 1998) and vaccinia virus (SPI-2) (Dobbelstein & Shenk, 1996).

The bcl-2 family members are also targeted to prevent apoptosis. Several viruses encode homologues of the antiapoptotic molecule bcl-2, for example KSHV (ORF16) (Sarid *et al.*, 1997) and EBV (BHRF 1 and BALF-1) (Henderson *et al.*, 1993; Marshall *et al.*, 1999). The expression of cellular bcl-2 members can also be modulated by viruses, for example EBV latent membrane protein (LMP) -1 upregulates the expression of cellular Bcl-2 (Henderson *et al.*, 1991).

Many viruses also encode molecules that target co-ordinators of the cell cycle such as p53, a molecule which promotes apoptosis; for example the product of the E6 gene of HPV binds p53 and targets it for ubiquitin-dependent proteolysis (Scheffner *et al.*, 1993).

Related to the subject of apoptotic death is another immune evasion mechanism by which virus-infected cells avoid CTL-mediated attack. This is by counterattack and triggering the death of virus-specific T cells. For example, HTLV-1 and measles virus are thought to counterattack virus-specific cells using pathways of death initiated by FasL and TRAIL (Xu *et al.*, 2001).

To summarise, viruses utilise a whole variety of strategies, targeting all arms of the antiviral response, to avoid detection and clearance by the host immune response. This allows such viruses to coexist with the host and often establish persisting infection. Viruses which are best able to persist in their hosts often use more than one type of immune evasion strategy. As described in sections

1.6 and 1.7, HIV uses multiple strategies to evade both the humoral and cell-mediated antiviral immune responses and to establish a persistent infection.

1.6 The HIV-specific immune response

HIV stimulates all the arms of the host immune response discussed in section 1.4. Here, each component of the anti-viral immune response is discussed in more detail, highlighting some of the experimental evidence which contributed to our understanding of the mechanisms used by the host to contain HIV replication. Despite the induction of the several arms of attack against the virus, the virus is able to persist in the host. The different ways in which the immune response is perturbed in HIV infection and immune evasion strategies used by HIV that contribute to virus persistence are also discussed here.

1.6.1 The innate response

(a) Type I IFNs in the host immune response to HIV

Elevated levels of type I IFN can be transiently detected in the serum during acute HIV-1 infection (von Sydow *et al.*, 1991). Peak serum type I IFN levels are observed prior to the peak in acute viral replication – IFNs may therefore have a role in containing viral replication during the initial stages of viral infection. By analogy with other virus infections, it is likely that PDCs play an important role in type I IFN production during primary HIV-1 infection. IFN production may be triggered by viral nucleic acids; and gp120 has also been implicated in the induction of IFN (Francis & Meltzer, 1993; Capobianchi *et al.*, 1992).

In mid-stage infection, levels of type I IFN in the serum are below detectable levels, so the contribution of type I IFNs to immune control of HIV during this stage is unclear. However this does not rule out the possibility that local type I IFN is produced in tissues and might play a role.

As disease progresses, IFN-α is again detected in the serum of infected individuals. Significant titres of IFN-α were found in the serum of patients who had progressed to AIDS (Grunfeld *et al.*, 1991; Kunzi *et al.*, 1995), including homosexuals with Kaposi's sarcoma (DeStefano *et al.*, 1982; Krown *et al.*, 1991). However, this was an unusual acid-labile form of IFN-α, raising questions about its efficacy. It has been suggested that production of this form of IFN may in fact contribute to HIV replication and disease progression (Hess *et al.*, 1991).

Cell culture models support a role for type I IFNs in limiting HIV infection. Type I IFNs have been shown to exhibit antiviral activity against HIV replication *in vitro* (Hartshorn *et al.*, 1987; Pitha, 1994). IFN-α administered at the time of viral challenge is able to block infection of monocytes (Francis *et al.*, 1992), and it is also able to suppress HIV expression in chronically infected cell lines (Poli *et al.*, 1989). Type I IFNs may indirectly contribute to suppression of HIV replication by inducing lysis of infected cells by NK cells (Biron, 2001). Other actions of IFNs may also contribute to an overall enhanced antiviral immune response, such as enhancing survival of PDCs (Cella *et al.*, 2000; Kadowaki *et al.*, 2000); promoting monocyte differentiation into potent antigen-presenting DCs (Lapenta *et al.*, 2003); and enhancing cross-presentation of viral antigens (Le Bon *et al.*, 2003).

The beneficial effects of type I IFNs are further highlighted by the therapeutic use of IFN-α in HIV-infected individuals. IFN-α was found to have efficacious antiretroviral and anti-tumour effects against KS (de Wit *et al.*, 1988; Lane *et al.*, 1988; Krown, 1998). Also, in a trial to test the clinical efficacy of IFN-α in patients with asymptomatic HIV infection, it was found that treatment of patients during primary HIV infection with IFN-α can result in a decrease in the frequency of virus isolation, suggesting a potential role in slowing down the progression of disease (Lane *et al.*, 1990).

The fact that administration of exogenous type I IFNs has a beneficial effect in HIV infection suggests that the production of type I IFNs may be compromised in infected individuals. Notably, the number of PDCs in the blood is generally reduced in HIV infection, both in primary infection (Kamga *et al.*, 2005; Pacanowski *et al.*, 2001; Soumelis *et al.*, 2002) and chronic infection (Feldman *et al.*, 2001; Soumelis *et al.*, 2001). It is unclear whether this is due to PDC loss, or a redistribution of these cells to LNs or other sites. However in addition to the numerical reduction in circulating PDCs there are also functional deficiencies too, for among those cells that remain, there is a lower frequency of responding cells (Feldman *et al.*, 2001). A strong positive correlation between IFN production and PDC count has been observed in control subjects but not in HIV-infected patients, who have lower IFN production on a per cell basis (Kamga *et al.*, 2005). The ability of PDCs from HIV-infected patients to stimulate allogeneic T cell proliferation in a mixed lymphocyte reaction (MLR) was also shown to be impaired (Donaghy *et al.*, 2003). Both the decrease in

number and the dysfunction of PDCs likely contribute to a reduction in IFN-α production by PDCs (Feldman *et al.*, 2001).

The plasma viral load in subjects has shown to be negatively correlated with IFN production or the number of functional PDCs (Donaghy *et al.*, 2001; Feldman *et al.*, 2001; Pacanowski *et al.*, 2001; Siegal *et al.*, 2001; Soumelis *et al.*, 2001). Also, IFN-α generation and PDC counts are recovered during HIV-suppressive therapy (Kamga *et al.*, 2005; Siegal *et al.*, 2001), suggesting that viral burden is inversely related to IFN-α generation. There is thus a direct correlation between the number of PDCs in the blood, IFN-α production and the clinical status of subjects (Soumelis *et al.*, 2001). PDCs are greater in number and also have increased function in long term non-progressors (LTNPs) relative to that found in progressors, AIDS-patients, and healthy controls (Soumelis *et al.*, 2001; Almeida *et al.*, 2005; Levy *et al.*, 2003). Numbers of PDCs and IFN production are markedly reduced in AIDS patients developing opportunistic infections and cancers (Feldman *et al.*, 2001; Lopez *et al.*, 1983; Siegal *et al.*, 2001; Siegel *et al.*, 1986; Soumelis *et al.*, 2001).

Infection of PDCs by HIV may represent a potential mechanism to account for the loss of PDCs in infected individuals. PDCs express CD4 and the chemokine co-receptors used by HIV for entry, and as such, are susceptible to infection. It has been demonstrated that they can be infected with HIV *in vitro* (Fong *et al.*, 2002; Patterson *et al.*, 1999; Patterson *et al.*, 2001; Smed-Sorensen *et al.*, 2005), and the presence of provirus was detected in purified PDC samples from HIV-infected patients (Donaghy *et al.*, 2003).

Primary HIV isolates, unlike laboratory-adapted strains, display variable sensitivity to the effects of IFN-α *in vitro* (Kunzi *et al.*, 1995), hence it is possible that in addition to the production of IFNs being compromised in HIV-infected individuals, resistance to IFN control may also contribute to the ability of the virus to persist *in vivo.*

(b) NK cells in the host immune response to HIV

NK cells may have a role in limiting HIV replication through their cytotoxic activity against infected cells, and via the production of C-C chemokines and IFN-γ which can suppress HIV infection (Fehniger *et al.*, 1998; Oliva *et al.*, 1998; Biron & Brossay, 2001). That NK cells may protect against HIV is

supported by observations made in intravenous drug users who remained uninfected despite high-risk exposure. When compared with drug users who did become infected, their NK cells were found to have significantly greater lytic activity *in vitro* (Scott-Algara *et al.*, 2003). In addition, there is evidence that NK cells make an important contribution to control of viral replication in infected individuals. Co-expression of a particular NK cell receptor, KIR3DS1, and its cognate ligand, Bw4, with a particular amino acid at residue 80, has been shown to be associated with slow disease progression (Martin *et al.*, 2002).

There are a number of mechanisms by which NK cell control of viral replication may be impaired in HIV-infected individuals, including reduction of NK cell numbers and functional capacity, and viral evasion of NK cell recognition.

It has been shown that despite a reported elevation in NK cell numbers in primary infection (Alter *et al.*, 2005), NK cells are lost from the periphery thereafter and are functionally suppressed (Alter *et al.*, 2005; Sinicco *et al.*, 1993). This becomes more pronounced in individuals with progressive disease, with NK cell-mediated lysis and cell numbers being most severely depressed in patients with AIDS (Cai *et al.*, 1990; Ratcliffe *et al.*, 1994).

Impaired cytotoxic ability and reduced IFN-γ production by NK cells in HIV infected individuals is associated with alterations in the expression of activating and inhibiting NK cell receptors, with enhanced expression of inhibitory receptors and decreased expression of activating receptors (Mavilio *et al.*, 2003; Eger & Unutmaz, 2004; Ahmad *et al.*, 2001; De Maria *et al.*, 2003; Parato *et al.*, 2002; Fogli *et al.*, 2004). This is particularly evident in viraemic patients when compared to aviraemic patients (those in which viraemia had been suppressed to undetectable levels by antiretroviral therapy (ART)), suggesting that ongoing active viral replication has an important influence on NK cells. In agreement with this, receptor expression and cell number are returned to normal when viraemia is controlled with ART (Goodier *et al.*, 2003; Kottilil *et al.*, 2004).

There are also alterations in subpopulations of NK cells in HIV infection, with a selective reduction in the more cytotoxic $CD56^{dim}$ subset of NK cells, and an expansion of a functionally defective $CD56^{-}/CD16^{+}$ population of NK cells reported (Alter *et al.*, 2005; Oliva *et al.*, 1998; Scott-Algara & Paul, 2002; Tarazona *et al.*, 2002). These subset alterations are more pronounced in

viraemic patients compared to aviraemic or healthy donors (Mavilio *et al.*, 2003).

The causes of the perturbed NK cell numbers and function in HIV infection are unknown. The loss of NK cells may be in part a consequence of their participation in ADCC of gp120-coated CD4 cells, where concomitant loss of the CD4 targets and the NK cells by activation-induced cell death (AICD) or apoptosis occurs. The NK cells that do recover from the ADCC reaction show a loss of cytotoxic function, and acquire a $CD16^{dim/-}$ or $CD56^{dim/-}$ phenotype (Jewett *et al.*, 1997). Reduced numbers could also conceivably be due to direct infection of NK cells (a rare subset expressing CD4 and chemokine coreceptors for entry can be productively infected *in vitro* (Valentin *et al.*, 2002)), but this is unlikely to account for the significant reduction in numbers seen. What is perhaps most likely is that NK cell abnormalities may be secondary to other immune system defects in HIV-infected individuals; for example levels of IL-15, a cytokine involved in NK cell homeostasis, are reduced in HIV infection (Ahmad *et al.*, 2003).

Several HIV-encoded proteins have been shown to downregulate MHC class I expression in infected cells as a mechanism to avoid antigen presentation to HIV-specific $CD8^+$ T cells and thereby avoid CTL recognition. This could leave the cells susceptible to NK cell attack, since MHC molecules are recognised by inhibitory NK cell receptors, and act to deliver a negative signal to block NK cell function. However, expression of HLA-C molecules is retained on HIV-infected cells. The selective downregulation of MHC class I molecules by HIV, with downregulation of HLA-A and -B but not HLA-C, achieves a balance between minimising antigen presentation by MHC whilst still avoiding attack by NK cells (Cohen *et al.*, 1999).

(c) NK-T cells and $\gamma\delta$ T cells in HIV infection

NK-T cells and $\gamma\delta$ T cells may contribute to early containment of HIV replication since they can perform anti-viral functions such as lysis of infected cells, or producing cytokines and chemokines that interfere with viral transmission and/or replication or that activate other effector cells.

Peripheral blood NK-T cells are markedly depleted in HIV infected individuals, particularly in those with uncontrolled viraemia and marked $CD4^+$ T cell depletion. It is the $CD4^+$ subset of $V\alpha24$ NK-T cells that is selectively lost (Sandberg *et al.*, 2002). These NK-T cells express CD4 and chemokine

coreceptors, making them a target for infection by HIV (Motsinger *et al.*, 2002; Unutmaz, 2003), and this may explain their loss. Since they express Fas, their depletion may be potentially involve Fas-mediated apoptosis. NK-T cell depletion may be one factor which contributes to the increased susceptibility to tumours and opportunistic infections associated with development of AIDS (Unutmaz, 2003).

That $\gamma\delta$ T cells respond to HIV infection is evidenced by the lytic and proliferative responses observed against HIV-infected cells by Vγ9Vδ2 TCR T cell clones (Wallace *et al.*, 1996), where an expansion of cells is accompanied by a decline in p24 levels. Phosphoantigen-activated Vγ9Vδ2 T cells have also been shown to release chemokines with suppressive activity on HIV replication (Poccia *et al.*, 1999). The increase in the numbers of $\gamma\delta$ T cells at mucosal sites in a model of protective immunity in rhesus macaques challenged with SIV by a rectal mucosal route suggests that these cells may be important in protection in the initial stages of transmission of virus (Lehner *et al.*, 2000a). The mechanism by which protection is thought to be mediated is by chemokines generated by $\gamma\delta$ T cells preventing SIV infection by binding to CCR5 coreceptors (Lehner *et al.*, 2000a; Lehner *et al.*, 2000b).
A deletion of Vδ2 cells has been observed in HIV infection and those cells which remain are functionally anergic and frequently do not proliferate upon stimulation, perhaps as a consequence of chronic antigenic stimulation. IL-2 is not able to restore the responsiveness of these cells, which is mirrored by a defect in CD25 expression (Poccia *et al.*, 1996). The failure of these cells to produce IFN-γ and TNF-α cannot be restored by provision of IL-12 and IL-15 (Boullier *et al.*, 1999). However anergy can be reversed and functionality restored by HAART (Martini *et al.*, 2000); this may reflect the recovery of $CD4^+$ T cell helper function.

(d) The role of complement in HIV infection
Complement can be activated by HIV virions and may contribute to clearance of plasma virus. Activation of complement by HIV can result from direct interaction between C1q and specific sites in gp41, leading to activation of the classical pathway (Ebenbichler *et al.*, 1991; Stoiber *et al.*, 1994; Thielens *et al.*, 1993). Other routes to complement activation can be via the binding of antibody to plasma virus and activation of the alternative pathway (Sullivan *et al.*, 1996), and via the interaction of MBL with carbohydrate structures on the

viral glycoprotein leading to activation of the lectin pathway (Saifuddin *et al.*, 2000).

However, despite activation of complement and the resulting deposition of complement fragments on the viral surface, HIV is resistant to MAC-mediated lysis and can thus evade complement-mediated lysis (Stoiber *et al.*, 2001). HIV is intrinsically resistant to complement due to recruitment of negative regulators of complement activation to the virus surface, such as CD59, CD46, decay-accelerating factor (DAF), and factor H (Favoreel *et al.*, 2003; Stoiber *et al.*, 1996), which have a protective action.

HIV instead uses opsonisation with complement to its own advantage to enhance its spread. Opsonised virions can interact with CR-expressing cells, and these can be infected with high efficiency or retain viral particles on their surface, promoting transmission of virus to other permissive cells (Kacani *et al.*, 2001; Stoiber *et al.*, 2001). It has been shown that B cells in lymph nodes and peripheral blood expressing CRs can bind infectious immune complexes containing HIV, and this can facilitate infection of autologous T cells (Doepper *et al.*, 2000; Jakubik *et al.*, 1999). That a subset of CD4 T cells which expresses CRs is also more efficiently infected *in vitro* in the presence of complement represents another example of how HIV can use complement to facilitate transmission to CR-expressing cells (Delibrias *et al.*, 1993).

1.6.2 The adaptive immune response to HIV infection

HIV-1 also stimulates an HIV-specific immune response involving the development of antibodies, $CD8^+$ CTL and $CD4^+$ T helper cells (Figure 1.6).

(a) The HIV-specific antibody response

During primary HIV infection, envelope-specific IgG is detected before/at the time of the initial decline in plasma viraemia (Aasa-Chapman *et al.*, 2004; Ariyoshi *et al.*, 1992; Lindback *et al.*, 2000a; Lindback *et al.*, 2000b), 3-6 weeks following infection. However antibodies with neutralising activity are not generally detected until several months after infection (Aasa-Chapman *et al.*, 2004; Pilgrim *et al.*, 1997; Wei *et al.*, 2003; Legrand *et al.*, 1997), and so it was originally thought that it was unlikely that antibodies contribute to the control of viraemia during primary infection. A similar delay is seen in development of nAb is seen in SIV infection of rhesus macaques (Montefiori *et al.*, 1996; Reimann *et al.*, 1994). Although the antibodies produced during primary infection may not possess neutralising activity, it is possible that other effector

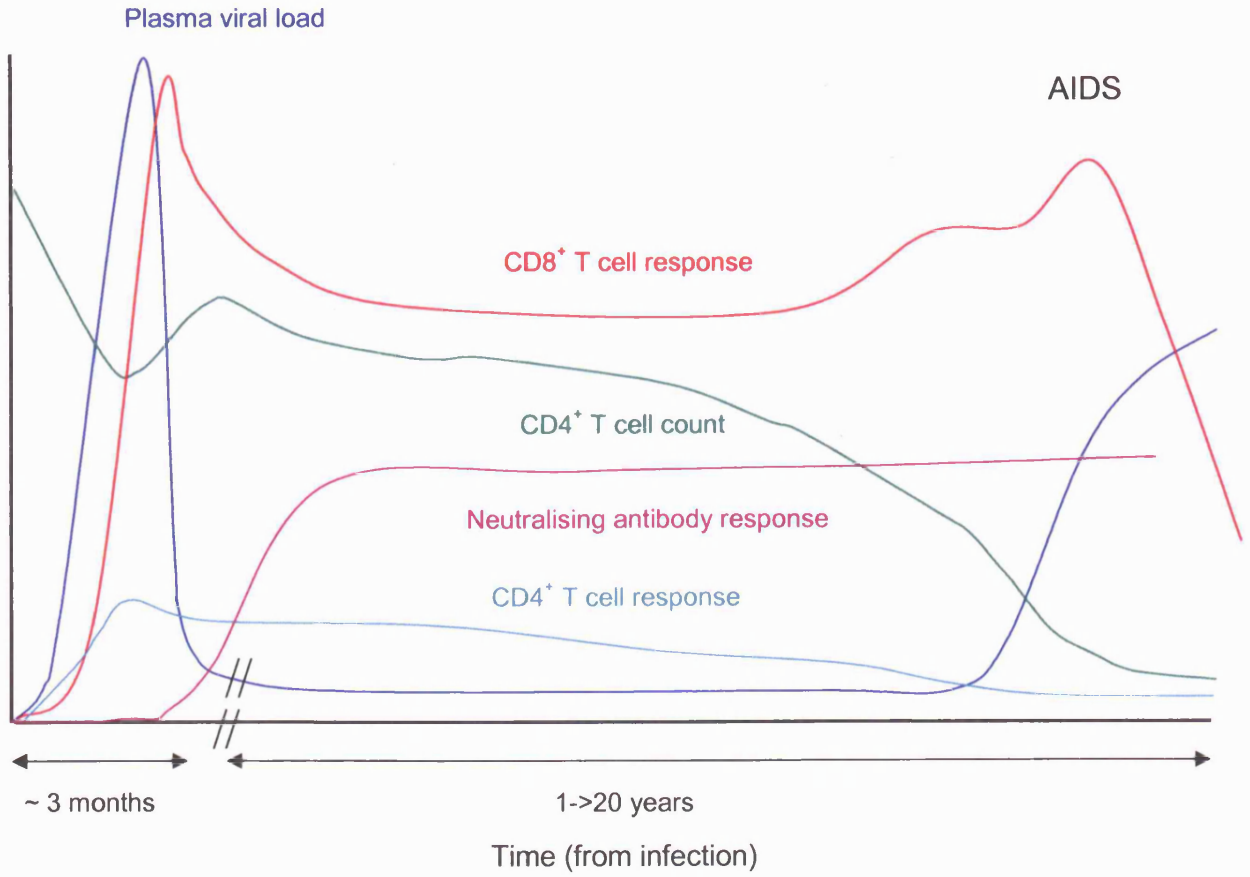


Figure 1.6. Diagrammatic representation of the HIV-specific immune response and the course of HIV-1 infection. The acute phase of infection is characterised by a burst of viral replication which is contained to a steady state level of persisting virus. Virus-specific $CD8^+$ T cell responses can be detected concomitant with containment of the acute viral burst; high levels of HIV-specific $CD8^+$ CTL are frequently detected during the chronic phase of infection and are thought to play an important role in control of viral replication. $CD4^+$ T cell responses are weak or undetectable throughout HIV infection in most individuals. Seroconversion occurs as or just after the acute burst of viral replication is contained, but the production of neutralising antibody may be delayed further. In end-stage infection there is a universal failure of host immunological function.

functions of antibody may play a role in controlling viraemia during primary infection (Aasa-Chapman *et al.*, 2004). In support of this, plasma or IgG from acutely infected patients was found to be able to inhibit the replication of primary HIV strains in the presence of NK cells. Antibody that was able to mediate ADCC was present in the majority of patients within days or weeks after the onset of symptomatic illness of primary infection (Forthal *et al.*, 2001). The temporal correlation in development of antibody with ADCC activity and decline in viraemia suggests that ADCC may play a role in control of viral replication in primary infection (Connick *et al.*, 1996). Furthermore, it has been recently demonstrated that antibodies to the viral envelope present during primary infection can inactivate virus by triggering the activation of complement (Aasa-Chapman *et al.*, 2005).

However studies in B cell-depleted animals suggested that antibody is unlikely to play a role in initial containment in acute viral replication in SIV infection, since in these antibody-deficient animals, a normal reduction in the level of viraemia was observed during the acute phase of infection (Schmitz *et al.*, 2003).

The reasons why the nAb response is delayed in primary HIV infection are unclear, but this may be partly attributed to deficits in the virus-specific $CD4^+$ T cell response during HIV infection, associated with which there may be a lack of B cell activation. Paradoxically, it has been shown that in LCMV-infected mice the presence of CD4 help may in fact impair the induction of nAb responses due to excess polyclonal B cell activation (Recher *et al.*, 2004). Another possibility is that B cells producing nAb may be destroyed by virus-specific $CD8^+$ T cells, as it is conceivable that the virus could bind to surface immunoglobulin on specific B cells and be internalised, then infecting these cells. The destruction of B cells displaying viral peptides by CD8 T cells has also been described in LCMV infection (Battegay *et al.*, 1993; Planz *et al.*, 1996).

HIV gp120, which mediates receptor binding, is the major target for nAbs. However, broadly neutralising antibodies are rare, especially those able to neutralise primary virus isolates. A number of structural features of the HIV envelope contribute to the characteristic resistance of HIV primary isolates to neutralisation (reviewed by (Pikora, 2004)).

i) The viral envelope is heavily glycosylated, with carbohydrate structures masking critical epitopes (Reitter *et al.*, 1998). Neutralising antibody epitopes

are contained in functionally conserved gp120 regions involved in binding to CD4 (CD4 binding site antibodies) or chemokine receptors (CD4-induced antibodies). However carbohydrates flank both of the receptor-binding regions on gp120, effectively shielding the virus from antibody-mediated neutralisation. In support of this, deletion of glycosylation sites which flank receptor-binding regions was shown to increase the sensitivity of primary virus to neutralisation by CD4 binding site antibodies and CD4-induced antibodies (Koch *et al.*, 2003). In SIV, elimination of N-glycosylation sites and a long deletion spanning variable loops V1 and V2 increased the sensitivity of the virus to neutralisation (Johnson *et al.*, 2003). Also, in an SIV model of infection where rhesus monkeys were infected with mutant forms of the virus lacking N-glycosylation sites in the envelope glycoprotein, antibody binding and neutralising activity increased (Reitter *et al.*, 1998).

ii) Overlapping hypervariable loops (such as V1 and V2) on the exposed surface of the viral envelope may also mask critical structural determinants (Parren *et al.*, 1999). These hypervariable regions of the envelope protein are immunodominant regions which can easily escape neutralisation.

iii) Many antibodies are directed against gp160 decoy proteins in cell surface debris or the monomeric soluble form of the envelope. These react poorly with the envelope as it exists in its oligomeric form on the surface of the virus and infected cells, as they are often directed against gp120 regions that are buried within the trimeric complex (Wyatt *et al.*, 1998).

iv) Neutralising antibodies may target regions of the envelope that are only transiently exposed at the time of virus entry, when the viral glycoprotein is ready to mediate fusion between viral and cellular membranes (Chan *et al.*, 1997; Weissenhorn *et al.*, 1997), by which time conformational constraints restrict the access of antibody molecules to conserved co-receptor binding sites on gp120. This is evidenced by the greater neutralising ability of Fab fragments than intact antibodies (Labrijn *et al.*, 2003).

When nAbs are raised *in vivo*, evidence suggests that they exert selective pressure on the virus as it continually and rapidly evolves to escape neutralisation. This accounts for some of the variation in the *env* gene observed in the early months after primary infection (Richman *et al.*, 2003). The escape virus is thought to acquire mutations involving changes primarily in N-linked glycosylation sites, such that an evolving glycan shield can prevent nAb binding but not receptor binding (Wei *et al.*, 2003). The emergence of

neutralisation escape mutants has also been reported in SIV infection (Burns *et al.*, 1993). Mutations in the gene encoding the surface glycoprotein with consequent nAb escape have also been observed in LCMV infection of mice. Here, the failure of the host to mount effective humoral responses against emerging neutralisation escape mutants correlated with the loss of $CD4^+$ T cell responsiveness during the establishment of persistent LCMV infection (Ciurea *et al.*, 2001), suggesting that impairment of $CD4^+$ T cell responses in HIV infection could also prevent the mounting of effective nAb responses to escape mutants.

(b) The HIV-specific $CD4^+$ T cell response

HIV-specific $CD4^+$ T cells can have direct antiviral effects by releasing antiviral cytokines and chemokines (such as IFN-γ, MIP-1α and MIP-1β) (Abdelwahab *et al.*, 2003; Lotti *et al.*, 2002; Norris *et al.*, 2001) or by directly killing infected cells (Littaua *et al.*, 1992; Lotti *et al.*, 2002; Norris *et al.*, 2004; Norris *et al.*, 2001). However, it is probably more through their helper functions for the antibody and CD8 response that they contribute to immune control of HIV. That patients with stronger HIV-specific CD4 responses do better, as demonstrated by low viral loads, highlights the importance of CD4 cells in control of viral replication. Examples of patient groups in which strong CD4 responses and low viral loads are observed are LTNPs (Rosenberg *et al.*, 1997) and HIV-2-infected subjects. Stronger virus-specific CD4 responses in HIV-2 compared to HIV-1 infection are associated with an undetectable viral load in the former infection (Berry *et al.*, 1998).

It is generally agreed that $CD4^+$ T cell function in blood is impaired early in infection. One study showed that HIV-specific $CD4^+$ cell responses could be transiently detected in primary infection (by antigen-stimulated release of IFN-γ), but then disappeared in untreated patients (Oxenius *et al.*, 2000) (probably due to the rapid destruction of activated $CD4^+$ cells in the acute phase of infection). Another study found that HIV-specific CD4 responses were sustained in patients who contained primary viraemia efficiently (Gloster *et al.*, 2004), consistent with which, the minority of patients who exhibit good immunological control of the virus were found to have vigorous polyclonal HIV-specific proliferative responses in chronic infection (Rosenberg *et al.*, 1997). Acutely infected patients treated with antiretroviral drugs also showed strong/preserved proliferative responses too (Harari *et al.*, 2004; Lichterfeld *et al.*, 2004; Rosenberg *et al.*, 1997). The majority of untreated HIV-infected

individuals exhibit poor CD4 proliferative responses to HIV and recall antigens *in vitro* (Clerici, 1989; Musey *et al.*, 1999). Some reports suggest that HIV-specific CD4 cell responses can be detected by intracellular IFN-γ staining after antigenic stimulation at all stages of infection (Pitcher *et al.*, 1999). However, in keeping with the general consensus that the number of responding HIV-specific CD4 cells is low, the frequency of HIV-specific cells in the latter study was found to be much lower than the frequency of CMV-specific cells in the same subjects.

The early dysfunction of HIV-specific helper T cell activity has been shown to persist, and HIV-specific responses are weak in the majority of chronically infected patients. The early impairment of proliferative responses to mitogens, recall antigens and HIV antigens is followed by improvement of responses to phytohaemagluttinin (PHA) and recall antigens within 6-9 months, but HIV-specific proliferation remains undetectable (Musey *et al.*, 1999). The lack of proliferative capacity of HIV-specific $CD4^+$ T cells in chronic infection is associated with diminished IL-2 secretion by these cells (Iyasere *et al.*, 2003; Younes *et al.*, 2003), suggesting a potential relevance of autocrine IL-2 secretion for maintaining HIV-specific $CD4^+$ T cell proliferative responses. In chronic infection HIV-specific $CD4^+$ T cell responses are generally inversely correlated with viral load (with stronger recall responses also being positively correlated with low viral load). Frequencies are higher in those with non-progressive disease (Pitcher *et al.*, 1999), and loss of CD4 function *in vitro* predicts progression to AIDS (Dolan *et al.*, 1995; Roos *et al.*, 1995).

The weak HIV-specific $CD4^+$ T cell response observed in the majority of HIV-infected individuals is likely due in large part to the destruction of HIV-specific $CD4^+$ T cells. HIV-specific $CD4^+$ T cells are ideal targets for infection by HIV, because they are activated as they respond to the virus. They may acquire infection from the APCs with which they interact during the activation process. Infected cells may either be destroyed by the lytic effects of replication or become targets for the $CD8^+$ CTL response. Cells may also be killed via ADCC triggered by antibody complexed with gp120 expressed on the surface of infected cells (Jewett *et al.*, 1997). There is also evidence that HIV selectively infects and eliminates HIV-specific $CD4^+$ T cells (Douek *et al.*, 2002). Other factors may lead to loss of $CD4^+$ cells besides that due to the direct cytopathic effects of the virus. Bystander killing may occur through Fas-mediated mechanisms during AICD or as a result of HIV proteins (e.g. Nef and

Vpr) released from infected cells, or inactivated virions stimulating apoptosis in uninfected cells (Alimonti *et al.*, 2003; Azad, 2000; Esser *et al.*, 2001). With the increasing cytokine dysregulation that is seen in progressive infection, the overproduction of cytokines such as IL-10 and IL-4 increases susceptibility to AICD.

Suppression of HIV-specific CD4 responses may also be attributed to other mechanisms besides their physical elimination. Env-specific CD4 responses are particularly lacking in the vast majority of patients. It was shown that HIV may evade induction of Env-specific responses by exploiting antibodies to suppress the processing of envelope antigens. gp120 presentation to $CD4^+$ T cells was inhibited by antibodies specific for the CD4 binding domain of gp120 (Hioe *et al.*, 2001); when gp120 was complexed to anti-CD4 binding domain antibodies, it was more resistant to proteolysis by lysosomal enzymes from APCs, and so peptide epitopes were not released and presented efficiently by MHC class II to gp120-specific CD4 cells (Chien *et al.*, 2004).

The activation and effector function of $CD4^+$ cells may also be impaired by effects of HIV infection on APCs, MHC class II-restricted antigen presentation and thus $CD4^+$ T cell priming. HIV Nef has been shown to downregulate the expression of peptide-loaded MHC class II molecules (Stumptner-Cuvelette *et al.*, 2001). Studies with other primate lentiviruses also show that Nef can modulate MHC class II expression on the cell surface (Schindler *et al.*, 2003). Co-stimulatory defects may also impact upon the development of HIV-specific T cell immunity. When dendritic cells infiltrating lymphoid tissue in acutely infected patients were characterised, the upregulation of co-stimulatory molecules that was seen in primary EBV infection did not occur in primary HIV infection (Lore *et al.*, 2002). Defects in the ability of APCs to present HIV antigens to T cell clones have also been suggested to be due to the binding of CD4 to APC-associated gp120, which may block/modulate the function of these cells (Fidler *et al.*, 1996). It is also thought that envelope proteins free in the circulation and bound to the surface of CD4 cells can interact with non-infected cells and have immunomodulatory effects on APCs. In addition, it is possible that infection and depletion of DCs may affect the stimulation of HIV-specific T cell proliferation (Donaghy *et al.*, 2003).

There is also evidence that the HIV-specific $CD4^+$ T cell responses that are present in HIV-infected individuals may be evaded by viral mutational escape. Variation in the envelope in viral isolates corresponding to regions that

stimulate helper T cell responses has been observed (Meddows-Taylor *et al.*, 2004). It was shown that HIV variants have the capacity to affect the activation of virus-specific $CD4^+$ cells (Harcourt *et al.*, 1998). In a rhesus monkey model, envelope variant peptides were still able to bind MHC class II molecules, but failed to stimulate the proliferation or cytokine secretion of Env-specific $CD4^+$ T cell lines (Lekutis & Letvin, 1998).

That HIV viraemia reduces the frequency of circulating HIV-specific $CD4^+$ cells may impact upon other help-dependent responses. For example, failure of T cell help could affect the ability of the immune system to deal with evolving viruses which have mutated to escape CTL and antibody responses that exert selective pressure on the virus. A less adaptable immune system that is unable to generate CTL/antibodies of new specificities may contribute to decline in immune control of the virus as disease progresses.

(c) The HIV-specific $CD8^+$ T cell response

$CD8^+$ T cells play an important role in the clearance or control of many viral infections. CD8 cells are able to inhibit viral replication by both cytolytic and non-cytolytic means (Buseyne *et al.*, 1996; Price *et al.*, 1998). Lysis is mediated predominantly by calcium-dependent exocytosis of perforin and granzyme proteases (Gulzar & Copeland, 2004; Shankar *et al.*, 1999; Smyth *et al.*, 2001), although a minority of CTLs use FasL to trigger apoptosis in target cells that also express Fas (Hadida *et al.*, 1999). HIV-specific $CD8^+$ T cells can also produce cytokines and chemokines that can affect viral replication. IFN-γ secreted from CD8 cells displays potent antiviral effects on HIV replication (Wells *et al.*, 1991), acting at an early step in the viral life cycle to reduce the synthesis of viral DNA (Meylan *et al.*, 1993). It is able to inhibit replication by affecting the expression or activity of cellular factors interacting with Tat, inhibiting Tat-induced transactivation of the HIV LTR (Emilie *et al.*, 1992). CC-chemokines such as MIP-1α, MIP-1β and RANTES are also produced by CD8 T cells (Price *et al.*, 1998; Wagner *et al.*, 1998) and released from cytotoxic granules. These suppress HIV replication by competition for, or downregulation of, the CCR5 coreceptor, thereby interfering with viral entry (Cocchi *et al.*, 1995). CD8 cells are also able to produce a soluble factor ($CD8^+$ cell derived anti-HIV-1 inhibitory factor (CAF)) that interferes with viral transcription, thereby shutting off virus production (Barker, 1999; Copeland *et*

al., 1995; Levy *et al.*, 1996). Alpha-defensins are thought to account for much of the anti-HIV activity of CAF (Zhang *et al.*, 2002a).

HIV has been shown to stimulate strong immune responses by $CD8^+$ T cells (Plata *et al.*, 1987; Walker *et al.*, 1987). During acute infection large expansions of oligoclonal $CD8^+$ T cells can be observed, with some reaching up to 40% of all T cells (Pantaleo *et al.*, 1994). Epitope-specific CD8 responses can be as large as 12% of $CD8^+$ cells (Doisne *et al.*, 2004). It has been estimated that to achieve this frequency starting from a naïve cell at a frequency of less than 1 in 10^6 requires 17 divisions over 2-3 weeks (McMichael & Rowland-Jones, 2001), illustrating the massive expansion that occurs in response to infection.

The clonal expansion of HIV-specific CD8 cells in response to an increasing antigenic load in acute infection is temporally associated with the resolution of primary viraemia (Borrow *et al.*, 1994; Koup *et al.*, 1994). The expanded CD8 population then partially contracts, as a consequence of apoptosis (Roos *et al.*, 1994; Selin & Welsh, 1994).

In chronic infection, the expanded HIV-specific T cells are maintained at high frequencies, when $CD8^+$ T cells targeting a dominant epitope can constitute 1-2% of all $CD8^+$ T cells (Altman *et al.*, 1996; Ogg *et al.*, 1998). This high frequency is maintained by continued antigenic stimulation of HIV-specific cells by viral replication, for it has been observed that when this viraemia is suppressed in HAART-treated patients, levels of HIV-specific responses decline (Draenert *et al.*, 2004a; Gray *et al.*, 1999; Kalams *et al.*, 1999a; Ogg *et al.*, 1999). Similarly in SIV-infected macaques, following the disappearance of the response due to limited antigenic stimulation, rechallenge with SIV caused CTL frequency to increase again (Geretti *et al.*, 1999).

During progression to AIDS, HIV-specific CTL responses deteriorate (Carmichael *et al.*, 1993; Greenough *et al.*, 1997; Hoffenbach *et al.*, 1989; Klein *et al.*, 1995), and this is typically paralleled by an increase in viral load.

In vitro studies have shown that HIV-specific $CD8^+$ CTLs can eliminate virus-infected cells via cytotoxic mechanisms (Yang *et al.*, 1996) and suppress viral replication by non-cytolytic mechanisms (Vella & Daniels, 2003). In addition to the observation that that HIV-specific $CD8^+$ CTL activity is temporally associated with the decline of plasma viraemia during acute infection (Borrow *et al.*, 1994; Koup *et al.*, 1994), there are other lines of evidence which also

suggest that HIV-specific $CD8^+$ T cells make an important contribution to the control of viral replication *in vivo*. The most direct evidence for the *in vivo* importance of CD8 cells in the control of HIV replication comes from studies of CD8-depleted monkeys that were unable to control viral replication in primary SIV infection (Jin *et al.*, 1999; Schmitz *et al.*, 1999). Also the selection of viral variants able to escape $CD8^+$ T cell recognition in both HIV infected individuals (Borrow *et al.*, 1997; Goulder *et al.*, 1997a; Price *et al.*, 1997) and SIV infected macaques (Allen *et al.*, 2000b; Evans *et al.*, 1999) suggests that the CD8 response is of *in vivo* importance and exerting a selective pressure on the virus. The inverse relationship between the $CD8^+$ T cell response and viral load (Klein *et al.*, 1995; Ogg *et al.*, 1998), and the loss of HIV-specific CD8 T cell responses with disease progression (Carmichael *et al.*, 1993; Klein *et al.*, 1995) also implicate CD8 cells with immunological control of HIV. The association between CD8 activity and a better disease status, with high levels of anti-HIV CD8 memory CTL in LTNPs (Greenough *et al.*, 1994; Harrer *et al.*, 1994; Harrer *et al.*, 1996; Rinaldo *et al.*, 1995), which are sustained (Klein *et al.*, 1995) also implies an important role for the CD8 response in controlling replication and preventing disease in such subjects. The influence of MHC genes on the course of infection (Kaslow *et al.*, 1996), with HLA alleles such as B27 and B57 being associated with slow and B35 being associated with rapid disease progression (Carrington *et al.*, 1999) also suggests that class I-restricted CD8 responses have an important influence. In addition, that resistant women who appear 'resistant' to infection despite repeated exposure to HIV show evidence of HIV-specific T cell responses further suggests that they may contribute to protection against infection (Fowke *et al.*, 2000; Kaul *et al.*, 2000; Rowland-Jones *et al.*, 1995; Rowland-Jones *et al.*, 1998).

1.7 Strategies used by HIV-1 to evade control by the HIV-specific $CD8^+$ T cell response

Despite the activation of strong CD8 responses which can reduce the initial acute viral burst of replication, HIV is not completely eliminated and it is able to replicate at high levels in the majority of infected individuals. The following discussion focuses upon how the virus might evade the HIV-specific $CD8^+$ response. The mechanisms involved fall into three categories as discussed in section 1.5.

1.7.1 Avoidance of recognition by the host immune response

HIV is able to avoid detection by the host immune response by establishing a latent infection in resting memory $CD4^+$ T cells and/or macrophages (and possibly other cell types) (Embretson *et al.*, 1993). In this situation, the viral genome (as cDNA) is integrated into the host genome, but is transcriptionally silent in the absence of activation and the associated lack of transcription factors. In this state, the virus is able to lay dormant and escape immune surveillance until cellular activation induces viral gene expression at a later time. This constitutes a stable viral reservoir that is a major obstacle to the eradication of virus using antiretroviral drugs.

HIV is also able to hide from the cellular immune response by establishing infection in anatomical locations to which T cells normally have no access. Such immunologically privileged sites in which HIV can be sequestered are also typified by limited MHC expression and an immunoregulatory environment, and include the brain, testes and eye. The latency and sequestration of HIV in anatomical and cellular reservoirs and the implications for eradication by antiretroviral therapy is reviewed in (Hamer, 2004; Persaud *et al.*, 2003; Saksena & Potter, 2003; Shearer, 1998; Wassef *et al.*, 2003).

Since high levels of productive viral replication also take place in sites readily accessible to the immune response, other mechanisms must be involved in evasion of the CD8 response. One way is through the virus avoiding antigen presentation to and recognition by CD8 cells by downmodulation of MHC class I. Three HIV proteins are able to reduce the expression of MHC on the surface of infected cells (reviewed in (Kamp *et al.*, 2000)).

Nef is able to cause selective internalisation of MHC molecules from the cell surface and thereby protect infected cells against CTL-mediated lysis (Collins *et al.*, 1998). Newly-synthesised MHC molecules are recruited into clathrin-coated pits and diverted towards the endosomal pathway where they accumulate in the trans-Golgi network and are degraded as they leave (Greenberg *et al.*, 1998; Le Gall *et al.*, 1998; Piguet *et al.*, 1998; Schwartz *et al.*, 1996). This downregulation of MHC is selective for HLA-A and HLA-B, since these contain a critical tyrosine residue in their cytoplasmic domain which is a necessary determinant for being able to respond to Nef (Le Gall *et al.*, 1998; Parham *et al.*, 1995). As already mentioned, the downregulation of MHC leaves cells susceptible to attack by NK cells. Infected cells still expressing HLA-C and HLA-E are however able to evade NK cell-mediated

destruction, since NK cells express receptors for HLA-C and HLA-E which inhibit NK cell activation (Cohen *et al.*, 1999).

HIV Tat and Vpu are also able to downregulate MHC by other mechanisms which act prior to Nef-mediated internalisation of MHC. Tat is able decrease the promoter activity of the class I gene (Howcroft *et al.*, 1993) by its interaction with a transcription factor (Weissman *et al.*, 1998), whereas Vpu is able to interfere with an early step in the biosynthesis of MHC class I molecules and induce the rapid loss of newly-synthesised class I heavy chains in the ER (Kerkau *et al.*, 1997).

Although *in vitro* studies show that a number of mechanisms act to reduce the expression of MHC class I, downregulation may not be complete. One *in vitro* study showed that although infection led to a 50% reduction in class I expression on the cell surface, lysis by CTL clones was unaffected (Yang *et al.*, 1996). By contrast, other studies have shown that MHC class I downregulation can impact in the efficiency of CTL lysis (Collins *et al.*, 1998). Therefore the extent of protection from CTL attack afforded by the downregulation of MHC class I molecules in infected cells *in vivo* is unclear.

One major way in which HIV can evade the virus-specific CD8 response is via antigenic variation. Mutational escape has been observed in other viral infections as a means of avoiding antibody or CTL recognition, but it seems to be particularly important in the case of HIV-1 (and SIV) infection.

It has been estimated that each time the 10kb genome of HIV is replicated, on average, one nucleotide substitution is introduced (Mansky & Temin, 1995). This is driven by two features of HIV replication which make the virus susceptible to mutational change: the high rate of viral replication, and the lack of proof-reading activity of the reverse transcriptase enzyme of this RNA virus. Any genetic change which results in a growth advantage will be rapidly selected for *in vivo*. In the face of the pressure exerted by the anti-viral $CD8^+$ T cell response on viral replication, antigenic variants which can escape the CTL response will be selected for as they will have a replicative advantage. However, there are constraints on immune escape (Wagner *et al.*, 1999). Mutations may arise that are in regions essential for replication, resulting in a virus that is unable to propagate itself (Nietfield *et al.*, 1995). So even though the mutations that arise may abrogate CTL recognition, they may also strongly impair viral replication. For the mutant virus to outgrow in the viral quasispecies, the benefit to be gained must outweigh any deleterious costs to

viral fitness. The capacity for mutations to be selected for in functionally important, more conserved regions of the genome, is therefore more limited.

The pressure exerted by the $CD8^+$ T cell response on an individual epitope will depend on both the magnitude and the efficacy of the response. Factors which determine the effectiveness of an epitope-specific response (and therefore influence the likelihood that escape variants will be selected for) may include the functional capacity of the epitope-specific T cells, and the avidity of recognition of the epitope by the T cells (O'Connor *et al.*, 2002). The timing of expression of the protein containing the epitope may also be important, for the kinetics of epitope expression may influence the effectiveness of HIV-specific CTL (van Baalen *et al.*, 2002; Yang *et al.*, 2003). Changes in dominant epitopes in early expressed proteins that elicit high avidity T cell responses may have a particularly great impact on immune control, and may thus be selected for rapidly.

Variation within a targeted epitope (or flanking residues) can confer escape from CTLs by altering epitope processing (Allen *et al.*, 2004; Del Val *et al.*, 1991; Draenert *et al.*, 2004b; Jones *et al.*, 2004; Yokomaku *et al.*, 2004), the ability of the epitope peptide to bind to MHC (Borrow *et al.*, 1997; Couillin *et al.*, 1994; Goulder *et al.*, 1997a; Price *et al.*, 1997; Rammensee & Monaco, 1994) or by altering the interaction of the MHC-peptide complex with the TCR (McAdam *et al.*, 1995; Rammensee & Monaco, 1994; Reid *et al.*, 1996).

Not only can the cytolytic activity of epitope-specific CD8 cells be impaired if a variant epitope fails to be recognised, for signalling through the TCR following antigen binding also results in chemokine release. Epitope variants that bound to HLA molecules but failed to stimulate cytolytic activity in CTLs were shown to also fail to stimulate chemokine release (Price *et al.*, 1998). Therefore antigenic variation might not only allow escape but might contribute to propagation of infection by its knock-on effect of reducing the inhibition of HIV entry by chemokines.

Furthermore, antigenic variation can have other effects on the T cell response besides escaping recognition by CTL. Epitope variants can induce anergy in T cells, antagonising the response to the original epitope, such that the response to the wild type virus is also impaired (Klenerman *et al.*, 1994). Altered interaction of the MHC-peptide complex with the TCR can also result in abnormal signalling, such that immunogenic variant peptides may induce the expansion of cells without effector function that are unable to control the mutant virus (McAdam *et al.*, 1995).

That CTL escape is a feature of HIV (and SIV) infection has been observed during various stages of infection. Escape from immunodominant CTL responses can occur very rapidly in acute/early infection (Borrow *et al.*, 1997; Price *et al.*, 1997). In primary HIV infection, the high virus turnover and large expansions of HIV-specific T cells may promote the rapid selection and fixation of escape mutants. Rapid CTL escape is also frequently seen in primary SIV infection (Allen *et al.*, 2000b; Evans *et al.*, 1999; O'Connor *et al.*, 2002; O'Connor *et al.*, 2003).

Selection for CTL escape viral variants has also been observed during mid-stage infection (Brander *et al.*, 1998; Goulder *et al.*, 1997b), but unlike during primary infection, mutations are frequently not selected for to completion within the viral quasispecies at this stage of infection.

Antigenic variation within an immunodominant epitope may shift responses to weaker epitopes. This may however weaken immune control over the virus because the subdominant CTL response may be less effective (Nowak *et al.*, 1995). Mutations can thus alter patterns of immunodominance. A complex picture of shifting hierarchies of immunodominance among CTL of different specificities and corresponding oscillations in the composition of the viral quasispecies emerges.

The frequency of occurrence of CTL escape during mid-stage infection may be lower than that in primary infection because of several factors:

i) The lower rate of viral replication at this stage, and a corresponding lower rate of mutation.

ii) The frequency of HIV-specific CD8 cells is lower than in primary infection, reducing the immune pressure exerted.

iii) Escape may be less common later on in infection as the CD8 T cell response may be broader than in primary infection (Draenert *et al.*, 2004a), reducing the advantage to be gained from escaping from any one component of the response.

iv) Any mutations that arise may be associated with high costs to viral fitness or else they would have already been selected for during primary infection.

v) The humoral response comes into play to contribute to control of viral replication, and so the advantage to gained from evading the CD8 response is not as great.

The selection for mutant forms of virus which escape CTL responses has also been seen during late infection in association with progression to AIDS (Goulder *et al.*, 1997a; Kelleher *et al.*, 2001), and also during late SIV infection

(Evans *et al.*, 1999). Escape at this stage of infection may ultimately contribute to the loss of control of replication as a declining immune system is less able to make new responses to new epitopes which emerge. In support of this, the emergence of an escape mutation during late infection was coincident with re-emergence of viraemia (Feeney *et al.*, 2004). However viral mutation may also be considered to be the result of increased viral replication. Mutations arising at this stage could be expected to be associated with high fitness costs to the virus. However, additional compensatory mutations can evolve over time to allow the outgrowth of the escape virus (Kelleher *et al.*, 2001) (although reversion to wild type sequences may still occur on transmission to new hosts, suggesting that compensatory mechanisms may only be partial).

Therefore mutational escape may play a major role in undermining CTL control of virus replication and the ultimate ability of the host to control the infection. In support of this, associations have been observed between immunodominant responses that are not escaped and good control of replication, as seen for a B57-restricted epitope (Migueles *et al.*, 2003). Furthermore, the limited emergence of escape viral variants in the context of good viral control compared to rapid selection for mutations in multiple epitopes in the setting of poor viral control has been reported (Jones *et al.*, 2004).

That CTL escape is common in HIV infection was demonstrated in a study of a large population of chronically infected patients, in which RT sequences from the study population were analysed (Moore *et al.*, 2002). Polymorphisms were most evident at sites of least functional constraint and a number were found to be associated with particular MHC class I alleles and to lie within epitopes known to be presented by these alleles. These 'footprints' provided evidence of adaptation by the virus to HLA-restricted immune responses at a population level.

CTL escape could influence the evolution of the virus in the population as a whole as well as within an individual, as evidence suggests that CTL mutations in certain epitopes can be stable when transmitted and accumulate (Goulder *et al.*, 2001b). In this study of the vertical transmission of virus encoding CTL escape variants in a dominant Gag epitope, an infected infant was found to target an otherwise subdominant B27-restricted epitope and failed to control replication. The escape variant remained stable without reversion in the absence of the pressure that originally selected for the mutation. Other studies, however, show that CTL escape variants may revert

to the wild type sequence *in vivo* in a new host in the absence of the original selective pressure (Allen *et al.*, 2004; Leslie *et al.*, 2004). In one case, despite the reversion back to wild type of one escape mutant within an epitope, another epitope mutation within the same epitope in the same patient was maintained (Leslie *et al.*, 2004). The costs to viral fitness exacted in escape may be an important factor which dictates reversion (Friedrich *et al.*, 2004).
Viral escape from CTL recognition may be a major limitation to CTL-based vaccines, as evidenced by the failure of an experimental AIDS vaccine as a result of escape from CTL recognition. Mutation within an immunodominant Gag epitope in animals vaccinated and then challenged with pathogenic simian-human immunodeficiency virus (SHIV) resulted in escape from the vaccine-induced response, a burst of viral replication and progression to AIDS (Barouch *et al.*, 2002).

1.7.2 Induction of a sub-optimal host immune response

Another explanation as to how HIV can persist in the face of the CD8 response would be that the response may not possess all the characteristics of an effective anti-viral response.
Analysis of $CD8^+$ T cell responses to many different viruses (e.g. LCMV, EBV, CMV and HCV) has suggested that effective CD8 responses are rapidly induced (Moskophidis *et al.*, 1987a), strong (Callan *et al.*, 1998b; Cooper *et al.*, 1999; Lechner *et al.*, 2000a; Murali-Krishna *et al.*, 1998; Wills *et al.*, 1996), broadly directed (Cooper *et al.*, 1999), functional (Gruener *et al.*, 2001; Zajac *et al.*, 1998) and sustained (Cooper *et al.*, 1999; Lechner *et al.*, 2000a). There have been relatively few studies of the virus-specific $CD8^+$ T cell response in acute/early HIV infection and it is currently unclear how well it conforms to these parameters. The following discussion reviews what is currently understood about various aspects of the nature of the HIV-specific CD8 response which could impact upon the efficiency of control of viral replication.

A number of studies have been carried out to investigate the magnitude of the HIV-specific $CD8^+$ T cell response in patients with different persisting viral loads to address whether this is one of the determinants of the efficiency of control of viral replication and disease prognosis. Different studies have come to different conclusions with regard to the relationship between the magnitude of the HIV-specific response and viral load. This may reflect the different methods used to quantitate HIV-specific cells, different patients cohorts used,

the stage of infection when the response was studied, or differences in the proteins studied.

Some studies have found an inverse relationship between HIV-specific CTL frequency and viral load (Edwards *et al.*, 2002; Greenough *et al.*, 1997; Ogg *et al.*, 1998; Rinaldo *et al.*, 1995; van Baalen *et al.*, 1997), supporting a role for HIV-specific CD8 responses in limiting replication. By contrast, several studies have found no relationship between the total magnitude of the HIV-specific $CD8^+$ T cell response and the viral load at different stages of infection and do not agree that high frequency responses are associated with better containment (Addo *et al.*, 2003; Betts *et al.*, 2001; Cao *et al.*, 2003; Dalod *et al.*, 1999a; Draenert *et al.*, 2004a; Gea-Banacloche *et al.*, 2000). This suggests some other parameters of the response may differentiate patients who contain viral replication well.

Another aspect of the CD8 response which may be important in determining its effectiveness is the epitope breath of the response, and the extent to which the overall response is biased towards a limited number of immunodominant epitopes. CTL escape is favoured if a particularly immunodominant response is made towards an individual epitope, as it is highly advantageous to the virus to escape from this response. It follows that escape would not be favoured in the setting of a broadly directed, co-dominant response targeting multiple epitopes where the immune pressure is spread out over a greater proportion of the viral genome, as escape from any one component of the response would not be as advantageous. The breadth of the HIV-specific CD8 response could thus impact upon the likelihood of the occurrence of CTL escape, and consequently the efficiency of control of HIV replication. That expansion of a response of limited breadth was associated with poor control of early viral replication was shown by the analysis of distortion of the T cell repertoire during primary infection in patients who established different persisting viral loads (Pantaleo *et al.*, 1997b). The implications of mounting responses of different breadths on CTL escape are also highlighted by a small study in which the breadth of the primary CTL response and extent of escape were compared in patients with differing persisting viral loads. It was found that escape was indeed restricted in a patient where there was a relatively broad, co-dominant distribution of CTL pressure, and that this was associated with establishment of a low persisting viral load (Jones *et al.*, 2004). Further studies are required to determine whether highly immunodominant responses

are more common in patients with poorer prognosis, and what factors contribute to the induction of a skewed response as opposed to a broad response.

Two studies have reported no relationship between the number of epitopes recognised and the viral load (Addo *et al.*, 2003; Masemola *et al.*, 2004) but these predominantly focused on chronically-infected patients, and neither addressed the relative dominance of epitope-specific responses in the study populations. Notably, one study suggested that that the primary response in many patients may be of limited specificity, as responses were detected to fewer epitopes in patients with primary infection compared to those with chronic infection (Dalod *et al.*, 1999b), although it is possible that this was due to differences in the specificity of the response in primary compared to chronic infection (Goulder *et al.*, 2001a), with the set of epitope peptides used to assess response breadth in the former study representing epitopes typically recognised in chronic rather than primary infection.

In support of the breadth of the response influencing the efficiency of control of viral replication are studies which link the degree of HLA heterozygosity with the rate of progression to AIDS (Carrington *et al.*, 1999). Individuals with the greatest degree of heterozygosity at HLA A, B and C loci would be able to present a wider range of epitopes, and consequently elicit responses of greater diversity and breadth.

In addition to the breadth of the response in terms of the number of T cells of different specificities, the clonality of individual epitope-specific responses may also be important. Epitope-specific responses which are dominated by limited numbers of T cell clones have been associated with poorer control of replication, implying that clonal breadth may be important in influencing control (Kalams *et al.*, 1994). Limited TCR diversity against an immunodominant epitope may limit recognition of virus variants with mutations in regions interacting with this TCR, thereby facilitating escape. The greater degree of clonality of the virus-specific CD8 response in HIV-2 infection may contribute to the better control of virus in HIV-2 infection compared to HIV-1 infection (Lopes *et al.*, 2003). The relatively limited breadth of TCR usage of HIV-1 epitope-specific CD8 cells was found to be associated with a less flexible response in terms of the ability to respond to amino acid substitutions within these epitopes. By contrast, the more diverse TCR usage of HIV-2-specific CD8 cells was associated with a greater ability to cross-recognise epitope variants and proliferate and produce IFN-γ. Polyclonal responses may

therefore have an enhanced capacity to tolerate mutations within epitopes. However, the preferential utilisation of specific TCRs may also be advantageous. For example a single TCR may be able to recognise multiple variants of an epitope, limiting the chances that viral mutation will lead to loss of CTL reactivity against the variant epitope (Dong *et al.*, 2004).

TCR use may be also important in determining the avidity of interaction between $CD8^+$ T cells and the APCs/targets cells; studies have suggested that TCR affinity may impact on the efficiency with which $CD8^+$ T cells mediate control of viral replication (O'Connor *et al.*, 2002; Yang *et al.*, 2003).

The epitope specificity of the HIV-specific CD8 response may also be of importance in determining its efficacy. It is suggested that the timing of target cell recognition critically contributes to CTL effectiveness (Gruters *et al.*, 2002) so direction of responses against epitopes in proteins expressed early in the viral lifecycle may be beneficial. Rapid epitope presentation on infected cells could allow infected cells to be detected by HIV-specific CD8 cells sooner, and allow a longer window of opportunity for CTL action before the release of viral progeny, and also before Nef-induced downregulation of MHC expression allows evasion of HIV-specific CTL. Proteins such as Rev, Tat and Nef are expressed early in the viral lifecycle, so CD8 responses directed epitopes within these proteins may be particularly efficacious. *In vitro* studies have supported this (van Baalen *et al.*, 2002; Yang *et al.*, 2003). Also, there is evidence for CTL responses directed against Tat being particularly effective in controlling *in vivo* virus replication. Reports of rapid escape from early protein-specific CTL during primary SIV infection (Allen *et al.*, 2000b; O'Connor *et al.*, 2001) imply that Tat-specific CTLs may be significantly involved in controlling replication and may have a high *in vivo* efficacy. Various studies have also shown that vaccine-induced responses against early proteins are associated with more efficient control of viraemia (Cafaro *et al.*, 1999; Maggiorella *et al.*, 2004; Stittelaar *et al.*, 2002).

There is evidence that the high cost of escape to intrinsic viral fitness may restrict viral escape from CTL responses to particular epitopes (Jones *et al.*, 2004; Kelleher *et al.*, 2001; Wagner *et al.*, 1999). The epitope specificity of the HIV-specific $CD8^+$ T cell response may thus also impact on the efficiency of control of virus replication in that responses which target functionally constrained regions of the genome (and hence are difficult for the virus to escape) may be advantageous.

Another parameter that may affect the efficiency with which HIV-specific $CD8^+$ T cells combat virus replication is their functional capacity. There have been questions raised about the functional capacity T cells for despite the induction of quantitatively large HIV-specific $CD8^+$ T cell responses, they are unable to mediate complete control over viral replication. In one study, it was found that the majority of HIV-specific CD8 cells in the peripheral blood contained low levels of perforin, reflected by poor *ex vivo* killing, when compared to CMV-specific cells within the same individual, which expressed high levels of perforin and killed well (Appay *et al.*, 2000). Low levels of perforin have also been reported in the CD8 cells in lymph nodes of infected patients (Andersson *et al.*, 1999; Trimble & Lieberman, 1998).

The effector function of $CD8^+$ T cells is related to their differentiation status, which is defined by the expression of specific cell surface markers. The perforin-deficient HIV-specific $CD8^+$ cells were shown to have lost CD28 expression but retain to CD27 expression. By contrast, CMV-specific cells lose the expression of both markers of cellular differentiation, marking them out as mature effectors (Hamann *et al.*, 1997), whereas the HIV-specific cells may be pre-terminally differentiated cells. In agreement with this, other studies using different markers show that the phenotype of HIV-specific cells ($CCR7^-$ $CD45RA^-$) lies between that of immature $CD8^+$ T cells ($CCR7^+$ $CD45RA^+$) and fully mature CD8 cells ($CCR7^-$ $CD45RA^+$, the phenotype of CMV specific CD8 cells) (Champagne *et al.*, 2001). The altered differentiation status of HIV-specific cells may thus affect their effector function as well as homing properties. Other features of the HIV-specific CD8 response suggestive of incomplete maturation include low *ex vivo* IL-2 production and proliferative capacity (Migueles *et al.*, 2002).

The reasons for inappropriate maturation of T cells and their dysfunction are not well understood, but may include impaired TCR signalling. CD3ζ, a signalling component of the TCR complex, was found to be downmodulated in HIV-specific CTL (Trimble & Lieberman, 1998). Its expression was shown to increase after culture in an IL-2-dependent manner, coincident with restoration of cytotoxic activity, suggesting that IL-2 is a co-factor necessary for proper activation of CD8 effector cells. In agreement with this, another study has shown that the deficits in effector function seen in SIV-specific CD8 cells in primary infection were reversible on extended incubation with IL-2 (Xiong *et al.*, 2001).

The failure of CD8 cells to mature could therefore be a consequence of impaired CD4 help, as CD4 T cells provide cytokines in addition to important cell-cell contact interactions with APCs needed for activation of CD8 cells. The positive correlation between levels of p24-specific proliferative responses and levels of Gag-specific CTL precursors (Kalams *et al.*, 1999b) highlights the association between CTL and helper responses. Further evidence to link APC function with CD8 function is the finding that CD28 co-stimulation increases the non-cytotoxic suppression of HIV replication in CD4 cells by CD8 cells (Barker *et al.*, 1997).

By affecting the function of CD4s and activation of APCs, and thus altering the pattern of cytokine production and engagement of cellular receptors, HIV may thus disrupt the signalling required for proper CD8 maturation. Improper TCR stimulation may promote the functional unresponsiveness of CD8 cells, and decrease the circulating pool of effector and memory CD8 cells able to combat infection.

Another consequence of impairment of CTL function is that it may result in higher rates of mutation owing to the higher level of viral replication due to poor control by dysfunctional cells. This may further favour the selection of escape variants and thus contribute to failure of immune control of viral replication.

1.7.3 Resistance to host effector mechanisms

HIV is also able to resist the effector mechanisms of HIV-specific cells. One way in which it does this is a consequence of the transition of the virus from the R5 type which utilises the CCR5 co-receptor, to the X4 type which uses the CXCR4 co-receptor during disease progression. A switch in co-receptor usage means that the virus will become insensitive to the effects to the CC chemokines released by activated CD8 cells, as they will no longer compete for the common receptor that they share.

The virus is also able to resist cytolysis by CD8 cells using a number of mechanisms. HIV is able to infect cells which may be inherently resistant to apoptosis, for example macrophages. It is thought that the differentiation of monocytes is accompanied by the upregulation of anti-apoptotic molecules and downregulation of pro-apoptotic molecules, resulting in the development of macrophages which are able to avoid destruction by CTL (Kedzierska & Crowe, 2002; Lum & Badley, 2003). HIV may also induce resistance to CTL in cells it infects. Studies of HIV proteins and their affects on apoptosis have

suggested that Nef, Vpr and Tat have regulatory effects on apoptotic processes (Badley *et al.*, 2000; Conti *et al.*, 1998; Xu *et al.*, 2001). Most notably, Nef has been shown to inhibit Fas and TNFR-1 signalling pathways through its interaction with ASK-1, a key signalling intermediate (Geleziunas *et al.*, 2001) and inhibition of caspase 3 and 8 activation (Yoon *et al.*, 2001). HIV Nef can also, via kinase activation, phosphorylate and inactivate bad, a pro-apoptotic molecule, and thereby block apoptosis in T cells (Wolf *et al.*, 2001). Infected cells can also 'fight back' against CTL by promoting apoptosis of $CD8^+$ CTL via the Fas pathway. Because HIV-specific $CD8^+$ T cells almost all express Fas, this renders them highly susceptible to Fas-induced apoptosis if they come into contact with cells expressing FasL. HIV Nef can mediate upregulation of the expression of FasL on the surface of infected cells (Xu *et al.*, 1997). This involves the interaction of Nef with the TCR zeta chain to form a signalling complex for the upregulation of FasL expression (Xu *et al.*, 1999), and results in the Fas-mediated apoptosis of virus-specific CTL that come into contact with infected cells. Another mechanism by which $CD8^+$ T cells can be triggered to undergo apoptosis is via TNF-induced death (Herbein *et al.*, 1998). The gp120 component of the HIV envelope can bind to CXCR4 on macrophages and induce the expression of TNF.

In summary, HIV is known to stimulate the induction of an innate response and virus-specific cell-mediated and humoral responses. Despite this, HIV is not cleared and is able to establish a persistent infection in the vast majority of infected individuals. This is likely due to multiple immune evasion mechanisms used by the virus. These may include weakened innate defence mechanisms, impairment, evasion and resistance to control by the humoral response, together with a variety of mechanisms for evading the anti-viral $CD8^+$ T cell response as discussed in detail here. Among the factors contributing to the difference in persisting viral load established in different HIV-infected individuals are likely to be the nature of the early antiviral immune response, and the extent to which this is evaded. A better understanding of the mechanisms involved, including the impact that defects seen in the HIV-specific $CD4^+$ T cell response during primary infection may have on the virus-specific $CD8^+$ T cell response, will help to facilitate the design of successful therapeutic and prophylactic strategies to combat HIV-1 infection and perhaps other persistent virus infections.

1.8 HIV-1 control, therapy and vaccine development

1.8.1 Control of transmission of HIV-1 infection

Several strategies to prevent HIV transmission are in use (reviewed in (Valdiserri *et al.*, 2003)). Globally, most cases of transmission result from unprotected sexual intercourse. Ways to prevent sexual transmission include promoting the adoption of safer sexual practises, use of microbicides and improving diagnosis and treatment of other sexually transmitted infections among those who are at risk for acquiring HIV, since it is known that untreated sexually transmitted infections can facilitate the transmission and acquisition of HIV (Wasserheit, 1992).

The screening of donated blood (and blood products) has virtually eliminated the spread of transfusion-associated HIV. Parenteral transmission in health care settings, for example occupational needle stick injury, can be avoided if safe working practises are used, treating blood and other bodily fluids from all patients as potentially infectious. The promotion of behavioural risk reduction and access to sterile needles has prevented the spread of HIV among injecting drug users.

Vertical transmission of HIV is estimated to be responsible for more than 90% of infections in children and infants (Quinn, 1996). Without treatment the risk of vertical transmission is 25-30%. In the developed world, the use of HAART can fortunately prevent the perinatal transmission of HIV in nearly 99% of cases (Minkoff, 2003). To prevent the transmission of HIV through breast milk, particularly in the developing world, breast milk formulas can be used.

Although such preventative interventions have been effective, in varying degrees, in curbing the spread of HIV, millions around the world are infected and so therapeutic strategies have to be employed.

1.8.2 HIV-1 antiretroviral therapy

As a result of the development of therapy, HIV has now become a treatable, chronic infection in the developed world. Several drugs targeting different parts of the viral life cycle are currently in use (reviewed in (Pomerantz & Horn, 2003)).

Introduced in 1987, zidovudine (azidothymidine (AZT)) was the first drug to be widely used. This is a nucleoside analogue which inhibits the HIV reverse transcriptase enzyme, termed a NRTI (nucleoside reverse transcriptase inhibitor). To enable viral replication to proceed, the ss RNA HIV genome is converted to ds DNA by reverse transcriptase. NRTIs competitively inhibit

reverse transcriptase by binding to the DNA chain and terminating its production, thus blocking viral replication.

Another class of drugs, the NNRTIs (non-nucleoside reverse transcriptase inhibitors) also target and inhibit the reverse transcriptase enzyme. These are a group of structurally diverse agents that, by binding at a distal position, induce conformational changes at the active site and effectively denature the enzyme.

Progress in drug development has led to the availability of new classes of drugs targeting different steps in the viral life cycle. The HIV Protease is responsible for the maturation of viral particles to infectious virions. Protease inhibitors bind to the active site of the enzyme and stop the protein cleavage events involved in the maturation of virions.

More recently fusion inhibitors were developed. These interfere with the fusion of the viral membrane with the cell membrane, thereby inhibiting viral entry. This involves preventing the full conformational changes in gp41 required for fusion.

Clinical trials are also underway to look at the clinical efficacy of integrase inhibitors and chemokine receptor entry inhibitors.

Initially the reverse transcriptase inhibitors were used as monotherapies. However, it was recognised that the effect of monotherapy was brief and limited. Although they did reduce viral load, their suppressive effect on viral replication was incomplete, and the high ongoing level of replication together with the selective pressure exerted by the drug enabled viral variants carrying mutations conferring drug resistance to emerge among the quasispecies (Concorde Coordinating Committee, 1994; Larder & Kemp, 1989; Volberding *et al.*, 1995).

With the increased availability of a wider range of other agents, various combination therapies were introduced. The triple combination therapy, or HAART, in use today effectively suppresses viral replication (Hirsch *et al.*, 1999), giving a more robust durable reduction in viral load (often to below the level of detection) and consequently limits the emergence of drug resistant variants. Furthermore, long term use of HAART is associated with the restoration of immune function, with the regeneration of robust CD4 and CD8 cellular responses to recall antigens (Autran *et al.*, 1997; Li *et al.*, 1998b).

However, there are several drawbacks associated with the use of antiretroviral drugs. The side effects of the drugs involve metabolic complications, including lactic acidosis, diabetes mellitus, lipodystrophy and pancreatitis (Carr *et al.*,

2000; Carr *et al.*, 1999; Carr *et al.*, 1998; Safrin & Grunfeld, 1999). The complicated regimens, requiring the intake of numerous tablets multiple times on a daily basis mean that there are adherence issues too. Poor adherence can result in an increase in viral replication, and facilitate the emergence of drug-resistant viral variants.

Despite the ability of HAART to reduce the plasma viral load to undetectable levels, obstacles to total eradication of the virus are presented by the low levels of replication that occur 'cryptically' in the presence of HAART (Dornadula *et al.*, 2001; Dornadula *et al.*, 1999) plus the existence of cellular reservoirs of latent infection, that can re-emerge after the withdrawal of therapy (Finzi *et al.*, 1997; Wong *et al.*, 1997). The timing of initiation of antiretroviral therapy is though to influence the level of residual viral reservoir, with early treatment found to result in a greater decline of HIV proviral DNA levels (Pires *et al.*, 2004).

So although combination antiretroviral therapy has been successful in significantly reducing morbidity and mortality in the developed world, issues of drug resistance and viral reservoirs remain. Furthermore, the use of such therapy where it is much needed in the developing world is hindered by the high costs and lack of medical expertise and infrastructure required to implement it.

1.8.3 HIV-1 vaccine development

As already discussed, there is an urgent need to develop prophylactic vaccines to prevent the further spread of HIV infection. There is evidence that the host immune response may in some cases prevent HIV. The demonstration in individuals who are reported to be persistently exposed to HIV yet seronegative of HIV-specific T cell and antibody responses (Broliden *et al.*, 2001; Devito *et al.*, 2000; Devito *et al.*, 2002; Kaul, 2001; Kebba *et al.*, 2004; Mazzoli *et al.*, 1997; Rowland-Jones *et al.*, 1995; Rowland-Jones *et al.*, 1998) suggests that vaccination might prevent or abort early infection. However despite intense efforts over the last twenty years, the development of an effective vaccine has proved difficult.

The majority of vaccines currently in use for protection against infection with different microorganisms work primarily through the induction of neutralising antibodies. However, the only HIV protein known to be a target for neutralising antibody is the HIV envelope and, as discussed earlier, the structure of the envelope makes it difficult for the host to generate antibodies capable of

neutralising primary HIV-1 isolates. The ideal vaccine would be one that induces sterilising immunity. The only likely way to achieve that, however, is through the induction of an antibody response that prevents infection upon exposure to HIV, ideally by neutralising a diversity of isolates. Because of the problems of stimulating HIV-specific neutralising antibodies, a number of the vaccine approaches currently being pursued are aimed at stimulating T cell responses, instead of or in addition to antibody responses.

Vaccines designed to stimulate CD4 and CD8 T cell immunity have been shown to confer some degree of protection on macaques from challenge with the aggressive SHIV89.6P strain (Amara *et al.*, 2001; Barouch *et al.*, 2000; Belyakov *et al.*, 2001; Rose *et al.*, 2001; Shiver *et al.*, 2002). Although the vaccinated macaques were infected, their viral load was dramatically reduced compared to that in unvaccinated controls.

Thus it is hoped that, although HIV-specific $CD8^+$ CTL cannot prevent the initial infection of cells, the induction of a strong T cell response will control early infection, reducing primary viraemia. This would have two major beneficial consequences: a lower initial viral load setpoint would enable the immune system to cope better so that the progression to AIDS is delayed, and also with a lower viral burden there is a lower likelihood of transmitting the virus. In addition, the role of CD4 cells in supporting the development of effective CD8 T cell and antibody responses (for example, in mice CD4 help has been shown to be crucial for priming an effective memory CD8 response (Shedlock & Shen, 2003; Sun & Bevan, 2003)) calls for the induction of helper responses to be an important requirement for an effective vaccine.

The first approaches to vaccine design were based on traditional strategies that had proven successful in preventing infection with other viruses. These involved the use of live attenuated viruses, inactivated viruses and viral components as subunit vaccines.

Live attenuated viral vaccines are generally very good at simultaneously inducing several immune effector mechanisms, and are in use for preventing polio, measles and varicella. The infection of monkeys with an SIV isolate made non-pathogenic by deletion of the gene encoding Nef was reported to protect monkeys from subsequent infection with wild type pathogenic SIV (Almond & Stott, 1999; Daniel *et al.*, 1992). However safety concerns about the long term stability of genetically altered viruses and the possible reversion to a pathogenic form, as seen in attenuated HIV (Greenough *et al.*, 1999;

Learmont *et al.*, 1999) and SIV (Baba *et al.*, 1995; Baba *et al.*, 1999; Whatmore *et al.*, 1995; Wyand *et al.*, 1997) strains, have restricted the utilisation of live attenuated HIV vaccines. For example, the late development of AIDS despite many years of disease-free infection in an Australian cohort infected by a single source of contaminated blood products was shown to be attributable to the re-emergence of a pathogenic form of a virus initially containing a mutation in the *nef* gene (Deacon *et al.*, 1995; Greenough *et al.*, 1999; Learmont *et al.*, 1999).

Inactivated virus vaccines have been used to elicit immunity that prevents infection with poliovirus and influenza. Despite an initially encouraging result in which monkeys were protected from an SIV challenge using a formalin-inactivated virus vaccine (Murphey-Corb *et al.*, 1989), this was later shown to be the result of induction of an immune response to antigens derived from the cells in which the virus was prepared (Stott, 1991). In light of this observation, the induction of allogeneic immune responses is being pursued as a possible approach to HIV vaccine design (Shearer *et al.*, 1999).

A recombinant protein vaccine for HIV has been sought in the hope of following the success of a vaccine against hepatitis B based on such technology. The first Env immunogens to be evaluated were monomeric recombinant envelope subunit vaccines. These have been tested in both non-human primate models (Berman *et al.*, 1990; Lubeck *et al.*, 1997) and in human volunteers (Cohen, 2003; Connor *et al.*, 1998; Graham *et al.*, 1998; Mascola *et al.*, 1996; Montefiori & Evans, 1999). The finding that recombinant gp120 protected against challenge with a homologous HIV-1 in vaccinated chimpanzees was met with scepticism owing to the use of immunogen and challenge virus with identical sequences. Results of the assessment of monomeric gp120 in human trials have been disappointing, revealing elicitation of antibodies which do not neutralise patient primary HIV isolates (Mascola *et al.*, 1996; Montefiori & Evans, 1999). Further, there were no benefits to the virological or clinical status of patients who were vaccinated with recombinant gp120 and subsequently became infected (Connor *et al.*, 1998). The first HIV vaccine candidate to progress to Phase III trials was a recombinant gp120 vaccine, and this failed to show any protective effect (see www.vaxgen.com).

Thus the application of traditional vaccine development strategies to HIV vaccine design has proved difficult. An important feature of inactivated viruses

and recombinant monomeric protein immunogens is that they do not elicit T cell responses very efficiently. While live attenuated vaccines stimulate strong CD4 and CD8 responses and neutralising antibodies, inactivated virus or purified protein subunit vaccines are less efficient, as they are poor at stimulating CD8 T cell responses, and the antibody responses induced frequently have restricted neutralising ability. Consequently, novel strategies are now being pursued in the hope of designing an efficacious vaccine.

Considerable efforts have been made to evaluate the potential use of live vector-based approaches and plasmid DNA immunogens. Genes encoding HIV proteins have been inserted into a variety of viral and bacterial vectors. Since their antigens are processed through the class I pathway, the immune responses to the HIV gene products include antibodies, helper T cells and CTL. For example, modified vaccinia Ankara (MVA), a poxvirus which has a number of large genetic deletions that attenuate its replicative potential in primate cells, is amenable to the insertion of HIV genes, which, when presented in this way have proved immunogenic (Barouch *et al.*, 2001; Hirsch *et al.*, 1996; Ourmanov *et al.*, 2000; Seth *et al.*, 2000). A variety of other viral (e.g. canarypox (Egan *et al.*, 1995; Pialoux *et al.*, 1995), Venezuelan equine encephalitis virus (Caley *et al.*, 1997), Semliki Forest virus (Hanke *et al.*, 2003; Mossman *et al.*, 1996) (Nilsson *et al.*, 2001) and adenovirus (Ferrari *et al.*, 1997; Shiver *et al.*, 2002; Vinner *et al.*, 2003)) and bacterial (e.g. BCG (Yasutomi *et al.*, 1993) and salmonella (Fouts *et al.*, 1995; Hone *et al.*, 1996)) vectors are also being developed as recombinant HIV vaccines. However, one concern is that their effectiveness may be limited by pre-existing host immunity to the vector, such as may be the case for vectors based on common serotypes of adenovirus (Sumida *et al.*, 2004).

DNA immunisation involves inoculation with plasmids encoding pathogen antigens. This results in protein production in local transfected cells as well as in directly transfected APC, which migrate to regional lymph nodes, eliciting both humoral and cellular responses to the specific immunising antigens.

The immunity elicited by DNA vaccines has demonstrated to be sufficient to reduce viral replication in monkeys subsequently challenged with SIV and SHIV (Barouch *et al.*, 2000; Egan *et al.*, 2000). Furthermore, monkeys immunised with DNA vaccines augmented by plasmid IL-2 constructs were protected against SHIV disease (Barouch *et al.*, 2000). The use of genes coding for cytokines is one strategy employed to enhance the efficacy of plasmid DNA vaccination. Other 'genetic adjuvants' encoding chemokines or

co-stimulatory molecules could also be useful. Inclusion of CpG motifs as a vaccine adjuvant to enhance the induction of immune responses has also been explored. Unmethylated CpG motifs in the DNA sequence have the capacity to mimic the ability of microbial DNA to stimulate innate responses which, in addition to limiting early viral spread, also promote the development of adaptive responses. Multiple delivery of the DNA vaccine and attempts to increase cellular uptake of DNA have also been explored as methods to boost responses (Estcourt *et al.*, 2004).

The most effective means of augmenting responses induced by DNA vaccines has been their incorporation into heterologous prime-boost immunisation regimes, taking into consideration that plasmid DNA represents a good priming immunogen, but that DNA plasmids may require relatively large doses to be immunogenic.

Vaccine design has thus progressed onto the use of bimodal vaccine regimens in which one vector (often plasmid DNA) is used to prime the immune response and a second (often live recombinant vector) is later used to boost that immunity. The use of two different vaccine vectors encoding a common immunogen has been recognised as a highly effective method for the induction of CTL activity in many models, with responses to the common vaccine antigen(s) but not vector antigens being boosted after the subsequent immunisation (Hanke *et al.*, 1998; Schneider *et al.*, 1998). The ability of DNA/poxvirus combinations to induce enhanced levels of CD8 T cell mediated protection has been attributed to the relatively low levels of antigen expression from the DNA vector facilitating the induction of higher avidity T cells which are then expanded by the booster immunisation (Estcourt *et al.*, 2002); and the induction of immune responses with enhanced IFN-γ production phenotypes or Th1 bias due to the cytokines induced by the vectors at the time of immunisation (Ramshaw & Ramsay, 2000; Woodberry *et al.*, 2003).

Prime-boost regimes (particularly DNA immunisation followed by boosting with poxvirus vectors) have been shown to stimulate strong CD8 responses and confer significant protection against pathogenic challenge with SHIV in rhesus macaques (Allen *et al.*, 2000a; Amara *et al.*, 2001; Hanke *et al.*, 1999; Robinson *et al.*, 1999; Shiver *et al.*, 2002). A number of Phase I and II trials involving prime-boost approaches are currently in progress.

Despite the current trend to pursue T cell-based vaccine approaches, research on ways to elicit HIV-specific neutralising antibody responses has not

been abandoned. There are a number of encouraging results from studies carried out using antibodies showing that antibody-mediated protection is possible. Vaccines based on the viral envelope can protect non-human primates challenged with homologous virus (Berman *et al.*, 1990; Emini *et al.*, 1992; Fultz, 1992; Girard *et al.*, 1995; Girard *et al.*, 1997; Lubeck *et al.*, 1997). Five broadly cross-neutralising human monoclonal antibodies have been isolated (Moore *et al.*, 2001). When passively transfused, these antibodies can protect severe combined immunodeficient (SCID) mice reconstituted with human lymphoid cells against challenge with HIV (Gauduin *et al.*, 1997) and can also protect monkeys against challenge with SHIV (Hofmann-Lehmann *et al.*, 2001a; Hofmann-Lehmann *et al.*, 2001b; Mascola *et al.*, 2000; Ruprecht *et al.*, 2001). Thus, induction of HIV-specific neutralising antibodies is possible in principle.

As mentioned earlier, unlike viruses adapted to laboratory culture, primary HIV isolates from infected patients are often resistant to neutralisation (Moore *et al.*, 1995). A number of approaches have been explored for engineering the structure of the HIV envelope with the aim of eliciting antibodies that may neutralise a variety of isolates. These approaches include the engineering of correctly folded and oligomerised Env immunogens that mimic the native structure of oligomeric Env (Binley *et al.*, 2000; Earl *et al.*, 1997; Earl *et al.*, 1994; Yang *et al.*, 2002); removing N-linked glycosylation and variable loop structures that may be responsible for masking neutralising epitopes (Barnett *et al.*, 2001; Binley *et al.*, 1998; Jeffs *et al.*, 1996; Quinones-Kochs *et al.*, 2002; Reitter *et al.*, 1998; Wyatt *et al.*, 1993); and triggering and stabilising conformational intermediates of gp120 and gp41 that arise during fusion, for example cryptic epitopes on gp120 that only become exposed after binding to CD4 (Fouts *et al.*, 2000), and the pre-fusion intermediate of gp41 (LaCasse *et al.*, 1999). However, none of these strategies have yet been successful in eliciting broadly neutralising antibody responses.

The antigenic opportunities for inducing CTL responses are not as limited as those for trying to elicit humoral responses, for the peptides recognised can originate from both surface proteins and inner structural and non-structural proteins, which may be relatively conserved, giving a greater number of protein targets.

Gag is usually included as an immunogen as seems to be very immunogenic in natural infection, contains important helper epitopes, and is also a relatively

conserved protein (Addo *et al.*, 2003; Betts *et al.*, 2001). Although they are quite variable proteins, Nef and Env are also often included as part of vaccine constructs. Given the kinetics of expression of these proteins, responses targeting them may be particularly efficacious.

One of the problems which HIV vaccine design faces is the enormous variability of the virus. It has been estimated that in theory, there is only a 1 in 3 chance that the T cell response stimulated by a vaccine of one clade will recognise the equivalent epitope from another clade (McMichael & Hanke, 2003). Potential solutions may be to include a combination of immunogens, possibly representing consensus clade sequences. It not only at the clade level where variability exists, and it is important to stimulate a response that is broad enough to cope with variability within clades too. This may necessitate splitting a multi-specific vaccine up into separate components rather than including them as one long protein so that the immune response sees each as a separate antigen against which to react and the immunodominance of certain epitopes is minimised (Palmowski *et al.*, 2002; Rodriguez *et al.*, 2002). Another reason for eliciting a broad response is that, as discussed earlier, by avoiding a narrow response, the danger that an escape mutant may be selected for is minimised. It has been shown that vaccine-induced immune control can be escaped by mutation (Barouch *et al.*, 2002).

Another important consideration in vaccine design is that high levels of immunity may need to be induced at mucosal sites to achieve protection against infection (Lehner *et al.*, 1999). The demonstration of the presence of HIV-specific neutralising antibody and CD8 responses in mucosal sites in HIV-exposed, seronegative individuals (Broliden *et al.*, 2001; Devito *et al.*, 2000; Devito *et al.*, 2002; Mazzoli *et al.*, 1997) highlights the importance of mucosal immunity in protection against a virus transmitted predominantly across mucosal surfaces.

Therapeutic immunisation, whereby HIV components are delivered to individuals with established infection in the hope of enhancing their immune responses to HIV, is another attractive goal of HIV vaccine research. Therapeutic immunisation may prove even more difficult than prophylactic vaccination, for inducing immunity in the face of multiple immunological defects acquired throughout the course of infection may be a great challenge.

Evidence that enhancement of HIV-specific immunity is possible comes from studies involving structured treatment interruptions (STIs) (Autran *et al.*, 1997; Autran *et al.*, 1999). When therapy is stopped, there is a rise in the level of replicating virus which stimulates immune responses, effectively allowing for autovaccination using autologous virus. Early treatment followed by an interruption has been associated with increased humoral and cellular responses and control of viral replication (Lisziewicz *et al.*, 1999; Ortiz *et al.*, 1999; Rosenberg *et al.*, 2000). However, STIs are not ideal as each burst of viral replication is associated with infection of more $CD4^+$ cells and undermining of the HIV-specific response. Hence, now attempts are being made to administer some of the vaccines discussed earlier in the context of HAART.

After many years of research, a number of HIV vaccine candidates are being evaluated in uninfected volunteers across the world (see www.iavi.org). These vaccine candidates (many of which primarily focus on the elicitation of cell-mediated immunity) present HIV immunogens in a number forms, including as recombinant proteins, inserts in viral vectors, plasmid DNA and synthetic peptides linked to lipid molecules, and are being tested in several combinations such as DNA serving as a prime for either protein or viral vector boosting. The majority of vaccine candidates are undergoing Phase I and II safety and immunogenicity trials, with one Phase III efficacy trial of a canarypox prime plus VaxGen gp120 boost in progress, results from which are not expected until 2008.

It is uncertain how responses observed in the primate models typically used for pre-clinical evaluation of potential vaccines will translate into actual protective efficacy in natural human infection. It is questionable as to whether they mimic human disease accurately, since the high dose challenges given to macaques to guarantee infection are probably far greater than the dose of HIV humans are exposed to during sexual contact. Immunisation strategies for eliciting strong, efficacious long term memory CD8 responses to vaccine antigens in humans need to be optimised; and strategies for eliciting nAb responses developed. Thus despite advances in the understanding of basic HIV biology and the host antiviral immune response, an effective protective vaccine still seems a distant prospect.

1.9 Project aims

As reviewed above, HIV-1 infection represents a major health problem across the world today, already responsible for millions of deaths and continuing to spread at an alarming rate. Although antiretroviral drugs which help reduce the viral load and delay disease progression are available, they do not eliminate infection, and are too expensive for use by the majority of infected individuals. Improved immunotherapeutic strategies and a prophylactic vaccine are thus urgently needed to control the AIDS pandemic.

One of the factors hampering HIV vaccine development is the current incomplete understanding of why, despite the induction of HIV-specific immune responses, in particular an apparently vigorous HIV-specific $CD8^+$ T cell response, the host is unable to eliminate the infection; and what kind of immune response vaccines should aim to elicit to provide good long-term control of virus replication.

The persisting viral load established during primary infection is thought to be an important prognostic indicator (Mellors *et al.*, 1996). Virus-specific $CD8^+$ T cell responses are induced in primary HIV infection (Borrow *et al.*, 1994; Koup *et al.*, 1994) and play an important role in control of early virus replication (Jin *et al.*, 1999; Schmitz *et al.*, 1999). The nature of the primary HIV-specific $CD8^+$ T cell response may thus be among the factors that determine the persisting viral load established. Despite its importance, there have been relatively few studies of the $CD8^+$ T cell response in acute and very early HIV-1 infection, and only limited information is available about its magnitude, kinetics, and epitope/clonal breadth or the phenotype and functional properties of the cells involved. Importantly, how these aspects of the response may differ between infected individuals - particularly individuals in whom primary viral replication is contained to differing setpoint persisting viral loads - is also unknown.

The aim of this project was therefore to characterise the primary HIV-specific $CD8^+$ T cell response in a number of infected individuals, to gain insight into quantitative, kinetic and qualitative features of the response, and if possible to determine their relationship to the persisting viral load established in primary infection.

The approach taken was to study the primary HIV-specific $CD8^+$ T cell response in patients from whom samples were available at sequential timepoints beginning prior to/during seroconversion and who did not receive antiretroviral therapy during acute/early infection. Important features of this

approach which have been reproduced in very few other studies include the ability to perform a longitudinal analysis of the virus-specific $CD8^+$ T cell response during acute and early infection, a critical period during which the virus-host dynamic is established; and the ability to analyse the relationship between features of the primary CD8 T cell response and the persisting viral load subsequently (naturally) established.

In chapter 3, I performed a "comprehensive" analysis of the epitope specificity of the $CD8^+$ T cell response in early infection in thirteen patients. Objectives here were to gain insight into the number and location of epitopes recognised and relative immunodominance of responses to each in early infection and to address the hypothesis that there may be differences in the epitope breadth, specificity and/or degree of biasing of responses towards immunodominant epitopes in patients who controlled viral replication with differing efficiency.

In chapter 4, I addressed the relationship between the functional avidity and relative dominance of epitope-specific responses in primary HIV infection, and looked for potential viral epitopes which may be cross-recognised by HIV-specific $CD8^+$ T cells. I also used murine models to investigate potential changes in the hierarchy of antiviral CD8 responses induced under conditions of limited CD4 help.
The aims here were to explore whether, when there is a paucity of CD4 help as occurs during primary HIV infection, responses involving T cells which are less affected by sub-optimal conditions of priming become particularly immunodominant in the primary virus-specific CD8 response. Such immunodominant responses were hypothesised to involve high avidity/affinity responses or memory cells cross-recognising epitopes from a heterologous virus.

In chapter 5, I used multimeric peptide-MHC complexes to identify HIV epitope-specific $CD8^+$ T cells at sequential timepoints over the course of primary infection to follow the kinetics of expansion of, and to assess the magnitude of, different individual HIV-specific responses. I also co-stained tetramer- (or pentamer-) positive cells with antibodies specific for other cell surface and intracellular markers to enable the extent of $V\beta$ family usage by T cells involved in individual responses and the phenotype of HIV-specific $CD8^+$ T cells to be followed over the course of primary infection.

By using tetramers specific for both immunodominant and subdominant responses and studying responses in patients with differing persisting viral loads, results of these experiments would enable the hypothesis that there may be differences in the kinetics, magnitude, clonality or phenotype of cells responding to different viral epitopes and/or in patients who control primary viral replication with differing efficiency to be addressed.

Chapter 2

Materials & Methods

2.1 Materials

2.1.1 Biochemical Reagents

Reagent	Supplier	Catalogue number
Acetonitrile	Sigma Aldrich Company Ltd, Poole, UK	A-3396
Agarose	Promega UK, Southampton, UK	V-3121
Ammonium sulphate	BDH Lab Supplies, Poole, UK	100334C
Bovine Serum Albumin (BSA)	Sigma Aldrich Company Ltd, Poole, UK	A4503
5'-Bromo-2'-Deoxyuridine (BrDU)	ICN Biomedicals Inc., Ohio, USA	100171
Concanavalin A (Con A)	Sigma Aldrich Company Ltd, Poole, UK	C-5275
Coomassie Plus 200	Perubio Science, Tattenhall, UK	23238
Crystal violet	BDH Laboratory Supplies, Poole, UK	3420244K
Cytofix/Cytoperm™ solution	Beckton Dickinson (BD) Biosciences Pharmingen, Oxford, UK	51-2090KZ
Dimethylsulphoxide (DMSO)	Sigma Aldrich Company Ltd, Poole, UK	D2650
Ethylene diamine tetra acetic acid (EDTA)	BDH Laboratory Supplies, Poole, UK	100935V
Ethanol	IAH Stores, Compton, UK	
Ethanolamine	Sigma Aldrich Company Ltd, Poole, UK	E9508
Formaldehyde	BDH Laboratory Supplies, Poole, UK	101136C
Glacial acetic acid	BDH Laboratory Supplies, Poole, UK	100015N
Heparin (Pump-Hep)	Leo Laboratories Ltd, Princes Risborough, UK	PL0043/0149

2-Mercaptoethanol	Sigma Aldrich Company Ltd, Poole, UK	M-7522
$Na^{51}CrO_4$ (^{51}Cr)	Amersham Pharmacia Biotech UK Ltd, Little Chalfont, UK	CJS11
Opti-Phase Supermix Scintillant	Wallac, Turku, Finland	SC/9235/21
Paraformaldehyde	Sigma Aldrich Company Ltd, Poole, UK	P6148
Perm/wash ™ buffer	BD Biosciences Pharmingen, Oxford, UK	51-2091KZ
PHA	Sigma Aldrich Company Ltd, Poole, UK	L-9132
Potassium chloride	Sigma Aldrich Company Ltd, Poole, UK	P4504
Sodium carbonate anhydrous	BDH Lab Supplies, Poole, UK	102405Y
Sodium hydrogen carbonate	BDH Lab Supplies, Poole, UK	102475W
Triton-X-100	Sigma Aldrich Company Ltd, Poole, UK	T9284
Tween-20	Sigma Aldrich Company Ltd, Poole, UK	P7949

2.1.2 Kits

Kit	Supplier	Catalogue Number
Chromogenic alkaline phosphatase substrate kit	BioRad Laboratories Ltd, Hemel Hempstead, UK	170-6432
QIAamp DNA blood mini kit	QIAGEN Ltd, Crawley, UK	51104

2.1.3 Plasticware

Product	Supplier	Catalogue Number
6 well plates	Becton Dickinson Labwear, NJ, USA	353046
24 Well Plates	Becton Dickinson Labwear, NJ, USA	353047
60mm cell culture dishes	Corning Costar, High Wycombe, UK	430166

100mm Petri dishes	Fahrenheit Laboratory Supplies, Milton Keynes, UK	351029
15ml Falcon tubes	Becton Dickinson Labwear, NJ, USA	352096
27G Needles	Sigma Aldrich Company Ltd, Poole, UK	Z19238-4
50ml Falcon Tubes	Becton Dickinson Labwear, NJ, USA	352070
96 flat bottom plates	Corning Costar, High Wycombe, UK	3595
96 well round bottom plates	Corning Costar, High Wycombe, UK	3799
Cell scrapers	Becton Dickinson Labwear, NJ, USA	3036
Cell strainers (40μm)	Becton Dickinson Labwear, NJ, USA	352340
Cluster tubes	Abgene, Surrey, UK	AB-0672
ELISPOT plates	Millipore, Watford, UK	MAHA S45 MAIP S4510
Eppendorf tubes 0.5ml 1.5ml	Tref Ag, Degersheim, Switzerland	96.4625.9.01 96.8160.9.02
FACS tubes (5ml round bottom polystyrene)	Becton Dickinson Labwear, NJ, USA	352054
Filters: 0.45μm	Millipore, Bedford, USA	16555
Flasks T150 T75 T25	Helena Biosciences, Sunderland, UK	90151 9076 9026
Glasstic® Slide 10 combination coverslip-microscope slides	Hycor Biomedical Ltd, Peniculk, UK	87144
Internal thread 1.8ml Nunc Cryovials	Life Technologies, Paisley, UK	377267
LD MACS columns	Miltenyi Biotech, Bisely, UK	130-042-901
Microscope slides	Merck Ltd, Lutterworth, UK	406/0184/04
Pasteur pipettes	Jencons Scientific, Leighton Buzzard, UK	475-068

Pipettes 5ml 10ml 25ml 50ml	Bibby Sterilin, Stone, UK Becton Dickinson Labwear, NJ, USA	40105 47110 18327 357550
Plate sealers	Camlab Ltd, Cambridge, UK	EC/005.SPN
Reagent reservoirs (100ml)	Corning Inc, NY, USA	4873
Single edge blades	TAAB Laboratories Equipment Ltd, Aldermaston, UK	B054
Slide-A-Lyzer (1000 MWCO) Dialysis Cassettes	Pierce & Warriner UK Ltd, Chester, UK	6638122
Syringes	Scientific Laboratory Supplies, Nottingham, UK	SYR 6200
Tips 20ul 200ul 1000ul	Rainin Instruments Co Ltd, Woburn, USA	GPS25 GPS250 GPS1000
Universal tubes	BDH Lab Supplies, Poole, UK	275/0460/04

2.1.4 Tissue Culture Reagents

Reagent	Supplier	Catalogue number
AIM V medium	Invitrogen Life Technologies, Paisley,UK	12055-091
DMEM (Dulbecco's MEM) with Glutamax medium	Invitrogen Life Technologies, Paisley,UK	31966-047
Foetal Calf Serum (FCS) (normal grade)	PAA Laboratories, Linz, Austria	A15-002 (Lot: 083)
Foetal Calf Serum (high grade)	Invitrogen Life Technologies, Paisley, UK	10106-169 (Lot: 40Q1282F)
Histopaque-1077	Sigma Aldrich Company Ltd, Poole, UK	H8889
Human Male AB serum	Sigma Aldrich Company Ltd, Poole, UK	H-4522
M199 medium	Invitrogen Life Technologies, Paisley, UK	21157-029

MEM (Eagle's) with Glutamax medium	Invitrogen Life Technologies, Paisley, UK	41090-028
Phosphate Buffered Saline (PBS)	Institute for Animal Health media supply	
Penicillin & Streptomycin	IAH Media Supplies, Compton, UK	
Red cell lysis buffer	Sigma Aldrich Company Ltd, Poole, UK	R7757
RPMI (Phenol-Red Free) Medium	Invitrogen Life Technologies, Paisley, UK	1835-030
RPMI 1640 with Glutamax medium	Invitrogen Life Technologies, Paisley, UK	72400-021
Saline	Baxter Healthcare, Compton, UK	F7124
Trypsin-EDTA	Invitrogen Life Technologies, Paisley, UK	45300-019
Tryptose phosphate broth	Institute for Animal Health media supply	
Water (Sterile)	Institute for Animal Health media supply	

2.1.5 Cell Lines

Cell Line	Supplier	Catalogue Number
BHK	Laboratory stocks, originally obtained from ATCC	
EBV-B-LCL (Patient BORI)	Dr P. Borrow, Compton, UK	
GK1.5	ATCC	TIB-207
RMAS cells	Shirley Ellis, Institute For Animal Health, Compton, UK	
143TK-	Laboratory stocks, originally obtained from ATCC	
Vero	Laboratory stocks, originally obtained from ATCC	

2.1.6 Cytokines

Cytokine	Supplier	Catalogue Number
Recombinant human IL-2	Roche Diagnostics, Lewes, UK	1011456

2.1.7 Antibodies

Antibody	Supplier	Catalogue Number	Clone	Isotype
Anti-human:				
CCR7 APC	R & D Systems Europe Ltd, Abingdon, UK	FAB197A	150503	IgG_{2a}
CD3	Harlan Sera Lab UK Ltd, Biscester, UK	Custom produced	OKT3	
CD3 FITC	Serotec, Kidlington, UK	MCA463F	UCHT1	IgG_1
CD4 MACS Beads	Miltenyi Biotech, Bisley, UK	130-045-101	SK3	IgG_1
CD8 MACS Beads	Miltenyi Biotech, Bisley, UK	130-045-201	BW135/80	IgG_{2a}
CD8 PerCP	BD Biosciences Pharmingen, Oxford, UK	345774	SK1	IgG_1
CD25 APC	BD Biosciences Pharmingen, Oxford, UK	555434	M-A251	IgG_1
CD27 FITC	BD Biosciences Pharmingen, Oxford, UK	555440	M-T271	IgG_1
CD28 APC	BD Biosciences Pharmingen, Oxford, UK	559770	CD28.2	IgG_1
CD28 FITC	BD Biosciences Pharmingen, Oxford, UK	555728	CD28.2	IgG_1

CD45RA APC	BD Biosciences Pharmingen, Oxford, UK	550855	HI100	IgG_{2b}
CD57 FITC	BD Biosciences Pharmingen, Oxford, UK	555619	NK-1	IgM
IFNγ (ELISPOT capture Ab)	MabTech AB, Nacka, Sweden	3420-3	1-D1K	IgG_1
IFNγ-biotin (ELISPOT detection antibody)	MabTech AB, Nacka, Sweden	3420-6	7-B6-1	IgG_1
IL7R-biotin	R & D Systems	BAF306		IgG
Ki67 FITC	BD Biosciences Pharmingen, Oxford, UK	556026	B56	IgG_1
Vβ1 TCR FITC	Serotec, Kidlington, UK	MCA1591F	BL37.2	IgG_1
Vβ2 TCR FITC	Serotec, Kidlington, UK	MCA1592F	MPB2D5	IgG_1
Vβ3 TCR FITC	Serotec, Kidlington, UK	MCA1700F	CH92	IgM
Vβ5.1 TCR FITC	Serotec, Kidlington, UK	MCA1699F	IMMU 157	IgG_{2a}
Vβ5.2 TCR FITC	Serotec, Kidlington, UK	MCA1611F	36213	IgG_1
Vβ5.3 TCR	Serotec, Kidlington, UK	MCA1698	3D11	IgG_1
Vβ6.7 TCR FITC	Perbio Science UK Ltd, Northumberland, UK	TCR2657	OT145	IgG_1
Vβ7 TCR FITC	Serotec, Kidlington, UK	MCA1594F	ZOE	IgG_{2a}
Vβ8.1/8.2 TCR FITC	Serotec, Kidlington, UK	MCA1696F	56C5	IgG_{2a}
Vβ9 TCR	Serotec, Kidlington, UK	MCA1595	FIN9	IgG_{2a}
Vβ11 TCR FITC	Serotec, Kidlington, UK	MCA1596F	C21	IgG_{2a}

Vβ12 TCR FITC	Serotec, Kidlington, UK	MCA1597F	VER2.32.1	IgG_{2a}
Vβ13.1 TCR FITC	Serotec, Kidlington, UK	MCA1608F	IMMU 222	IgG_{2b}
Vβ13.2 TCR	Gift from Dr Tao Dong, Oxford, UK		H132.8	IgG_1
Vβ13.6 TCR FITC	Serotec, Kidlington, UK	MCA1598F	JU-74	IgG_1
Vβ14 TCR FITC	Serotec, Kidlington, UK	MCA1599F	CAS 1.1.3	IgG_1
Vβ16 TCR FITC	Serotec, Kidlington, UK	MCA1600F	TAMAYA 1.2	IgG_1
Vβ17 TCR FITC	Serotec, Kidlington, UK	MCA1697F	E17.5F3	IgG_1
Vβ18 TCR	Serotec, Kidlington, UK	MCA1601	BA62	IgG_1
Vβ20 TCR FITC	Serotec, Kidlington, UK	MCA1602F	ELL1.4	IgG_1
Vβ21.3 TCR FITC	Serotec, Kidlington, UK	MCA1603F	IG125	IgG_{2a}
Vβ22 TCR FITC	Serotec, Kidlington, UK	MCA1604F	IMMU 546	IgG_1
Vβ23 TCR	Serotec, Kidlington, UK	MCA1605	AF-23	IgG_1
Anti-mouse:				
CD3 APC	BD Biosciences Pharmingen, Oxford, UK	553006	145-2C11	IgG_1
CD3 FITC	BD Biosciences Pharmingen, Oxford, UK	553062	145-2C11	IgG_1
CD4 ascites	Qbiogene, Ilkirch Cedex, France			
CD4 PerCP	BD Biosciences Pharmingen, Oxford, UK	553052	RM4-5	IgG_{2a}
CD8 Cy5	TCS Biologicals Ltd, Buckingham, UK	RM2211	CT-CD8a	IgG_{2a}

CD8 FITC	BD Biosciences Pharmingen, Oxford, UK	553031	53-6.7	IgG_{2a}
CD8 PerCP	BD Biosciences Pharmingen, Oxford, UK	553036	53-6.7	IgG_{2a}
Fc γ III/II receptor block (CD16/CD32)	BD Biosciences Pharmingen, Oxford, UK	553142	2.4G2	IgG_{2b}
H-2 D^b FITC	Serotec, Kidlington, UK	MCA1505F	CTD^b	IgG_{2a}
H-2 K^b FITC	Serotec, Kidlington, UK	MCA1503F	CTK^b	IgG_{2a}
IFN-γ (ELISPOT capture antibody)	MabTech AB, Nacka, Sweden	3321-3-1000	AN18	IgG_1
IFN-γ – biotin (ELISPOT detection antibody)	MabTech AB, Nacka, Sweden	3321-6-1000	R46A2	IgG_1
IgG beads	Dynal, Bromborough, UK	110.02		
Vβ TCR screening panel	BD Biosciences Pharmingen, Oxford, UK	557004		
Vβ2 TCR FITC			B20.6	IgG_{2a}
Vβ3 TCR FITC			KJ25	IgG (group 2)
Vβ4 TCR FITC			KT4	IgG_{2b}
Vβ5.1, 5.2 TCR FITC			MR9-4	IgG_1
Vβ6 TCR FITC			RR4-7	IgG_{2b}
Vβ7 TCR FITC			TR310	IgG_{2b}
Vβ8.1, 8.2 TCR FITC			MR5-2	IgG_{2a}
Vβ8.3 TCR FITC			1B3.3	IgG (group 3)
Vβ9 TCR FITC			MR10-2	Ig_{G1}
$Vβ10^b$ TCR FITC			B21.5	IgG_{2a}
Vβ11 TCR FITC			RR3-15	IgG_{2b}
Vβ12 TCR FITC			MR11-1	IgG_1
Vβ13 TCR FITC			MR12-3	IgG_1

Vβ14 TCR FITC Vβ17[a] TCR FITC			14-2 KJ23	IgM IgG_{2a}

Isotype control antibodies	Supplier	Catalogue Number	Clone
Mouse IgG_1 FITC	BD Biosciences Pharmingen, Oxford, UK	550616	MOPC-31C
Mouse IgG_1 PE	BD Biosciences Pharmingen, Oxford, UK	550617	MOPC-31C
Mouse IgG_{2a} FITC	BD Biosciences Pharmingen, Oxford, UK	554647	G155-178
Mouse IgG_{2b} FITC	BD Biosciences Pharmingen, Oxford, UK	33804X	27-35
Rat IgM FITC	BD Biosciences Pharmingen, Oxford, UK	553942	R4-22

Secondary step reagents	Supplier	Catalogue Number
Anti-biotin AP (alkaline phosphatase)	Vector laboratories, Inc., Orton Southgate, UK	SP-3020
Anti-mouse IgG/IgM (H+L)-FITC (Goat)	Stratech Scientific Ltd, Soham, UK	115-096-068
Anti-rat IgG beads	Dynal, Bromborough, UK	110.08
Steptavidin-Alkaline phosphatase	MabTech AB, Nacka, Sweden	3310-10
Streptavidin-APC	BD Biosciences Pharmingen, Oxford, UK	554067
Streptavidin-FITC	BD Biosciences, Pharmingen, Oxford, UK	13024D

2.1.8 Viruses

Recombinant vaccinia viruses expressing HIV genes used for *in vitro* assays:

Virus	Expresses	Supplied by	References
vSC8	Vaccinia strain WR encoding *E.coli* β-galactosidase (β-gal) gene under the control of the vaccinia P11 promotor.	Dr. B. Moss via the AIDS Research and Reference Reagent Program.	(Chakrabarti *et al.*, 1985)
vPE16	Vaccinia strain WR encoding the entire *env* gene of HIV-1 clone BH8 under the control of the vaccinia P7.5 promotor, plus *E.coli* β-galactosidase gene under the control of the vaccinia P11 promotor.	Dr. B. Moss via the AIDS Research and Reference Reagent Program.	(Earl *et al.*, 1990)
$vTFNef_2$	Vaccinia strain WR encoding the complete ORF *nef* of HIV-1 strain NL43 under the control of the vaccinia P7.5 promotor, plus *E.coli* β-galactosidase gene under the control of the vaccinia P11 promotor.	Dr. B. Moss via the AIDS Research and Reference Reagent Program.	(Koenig *et al.*, 1990)
vTat	Vaccinia strain WR encoding the *tat* gene of HIV-1 strain BH10 under the control of the vaccinia P7.5 promotor, plus *E.coli* β-galactosidase gene under the control of the vaccinia P11 promotor.	Dr. B. Moss via the AIDS Research and Reference Reagent Program.	(Falkner *et al.*, 1988)

vAbT-141.5.1	Vaccinia strain NYCBH encoding the *gag* gene of HIV-1 strain BH10 under the control of the vaccinia P7.5 promotor, plus *E.coli* β-galactosidase gene under the control of the vaccinia P11 promotor.	Dr. D. Panicali, Therion Biologics, Cambridge, MA, USA	(Koup *et al.*, 1989)
vP1288	Vaccinia virus strain WR encoding the entire *pol* gene of HIV-1 IIIB contained within the vaccinia K1L region. Purified by host range selection system.	Virogenetics Corp. via the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH	(Goebel *et al.*, 1990; Gurgo *et al.*, 1988; Perkus *et al.*, 1989; Ratner *et al.*, 1985)

Viruses used for murine infections:

Virus	**Expresses**	**Supplied by**	**References**
Influenza virus A/PR/8/34		Charles River SPAFAS, North Franklin, USA	
LCMV Armstrong		Dr. M.B.A. Oldstone, The Scripps Research Institute, La Jolla, USA	(Dutko & Oldstone, 1983)
Pichinde virus (PV) (strain AN3739)		Dr. M. J. Buchmeier, The Scripps Research Institute, La Jolla, USA	(Buchmeier & Rawls, 1977)

vvgplong	Vaccinia strain WR encoding the full-length GP gene of the Armstrong strain of LCMV under the control of the vaccinia P7.5 promotor, plus E.coli B-galactosidase gene under the control of the vaccinia P11 promotor.	Dr. J. Lindsay Whitton, The Scripps Research Institute , La Jolla, USA	(Whitton *et al.*, 1988)
vVNP	Vaccinia strain WR encoding the entire nucleoprotein (NP) gene of the Armstrong strain of LCMV under the control of the vaccinia P7.5 promotor, plus E.coli B-galactosidase gene under the control of the vaccinia P11 promotor.	Dr. J. Lindsay Whitton, The Scripps Research Institute , La Jolla, USA	(Whitton *et al.*, 1988)

2.1.9 MHC class I-peptide multimers

Multimer	Fluorphore	Supplier
H-2 D^b FQPQNGQFI tetramer	APC	ProImmune, Oxford, UK
H-2 D^b KAVYNTAC tetramer	APC	ProImmune, Oxford, UK
H-2 D^b WMHHNMLDI (Uty) tetramer	PE	Dr Liz Simpson, Hammersmith Hospital, London, UK
HLA -A*0101 GSEELRSLY tetramer	PE	Beckman Coulter, San Diego, USA
HLA-A*0201 YTAFTIPSI pentamer	PE	ProImmune, Oxford, UK
HLA-A*0301 QIYAGIKVK tetramer	PE	ProImmune, Oxford, UK
HLA-A*0301 QVPLRPMTYK tetramer	PE	ProImmune, Oxford, UK
HLA-A*0301 RLRPGGKKK tetramer	PE	ProImmune, Oxford, UK

HLA-A*1101 AAVDLSHFLK tetramer	PE	Beckman Coulter, San Diego, USA
HLA-A*1101 VPLRPMTYK tetramer	PE	Beckman Coulter, San Diego, USA
HLA-B*0702 IPRRIRQGL pentamer	PE	ProImmune, Oxford, UK
HLA-B*0702 RPQVPLRPMTY tetramer	PE	ProImmune, Oxford, UK
HLA-B*0702 TPGPGIRYPL pentamer	PE	ProImmune, Oxford, UK
HLA-B*0801 FLKEKGGL tetramer	PE	Beckman Coulter, San Diego, USA
HLA-B*0801 GEIYKRWII tetramer	PE	Beckman Coulter, San Diego, USA
HLA-B*3501 RPQVPLRPMTY tetramer	PE	Beckman Coulter, San Diego, USA
HLA-B*5701 KAFSPEVIPMF tetramer	PE	Beckman Coulter, San Diego, USA

2.1.10 Peptides

Synthetic peptides were supplied by Chiron Mimotopes (Clayton, Australia) in a peptide-amine-acid format or were synthesised by Larry Hunt (IAH, Compton, UK) using Fmoc or TBoc chemistry. Lyophilised peptides were resuspended in either DMSO or an organic solvent, pH modifier and water and stored at -20°C.

2.1.11 Mice

Female C57BL/6 mice and IA knock-out (k/o) mice 8-12 weeks of age were obtained from the SPF animal unit (IAH, Compton, UK) and Charles River (Margate, UK).

2.2 Methods

2.2.1 Human study subjects

HIV-infected patients: The majority of samples were obtained from a cohort of HIV seroconverters attending clinics at the Mortimer Market Centre (MMC) (London, UK) as part of a collaboration with Dr Ian Williams (Royal Free and

University College London Medical School, UK). The cohort consisted predominantly of Caucasian male homosexuals who reported to the clinic with acute retroviral symptoms (such as nausea, vomiting, headaches and fever) following a high risk exposure incident. Confirmation of the HIV status of the patient was made by enzyme-linked immunosorbent assay (ELISA). Samples from additional HIV seroconverters were obtained from Dr. George Panayiotakopoulos (St. George's Hospital, London, UK) and Dr. George Shaw (University of Alabama at Birmingham, USA). Samples used from patients chronically infected with HIV were obtained from Dr. Ian Williams. Ethical approval for these studies was obtained from the local ethical review/human subjects committees, and written informed consent for participation in the study was obtained from all patients. Viral load data, CD4 cell counts and clinical histories of the patients were supplied by Drs Pippa Newton, Pierre Pellegrino and Ian Williams (Royal Free and University College London Medical School).

Peripheral blood samples were collected from acutely-infected patients at sequential timepoints during and after seroconversion (at weekly intervals for the first month following onset of symptoms, at weeks 6, 8 and 12, and then every three months until two years, at which point samples were taken every six months). Samples used in our studies were taken at timepoints during which the subjects were not receiving any ART. All samples were processed on the same day of venepuncture. PBMCs were isolated and frozen and stored in liquid nitrogen until use.

HIV-seronegative subjects: As a source of control HIV-negative PBMCs, cells isolated from buffy coat bags were used. These were obtained from the North London blood transfusion service (Collindale, London, UK) and had been screened to confirm HIV-seronegativity before release.
For cross-reactivity studies, fresh blood from Donor JSS, a subject who was previously found to respond to the B44 restricted EBV epitope EENLLDFVRF, was provided by Dr A. Carmichael (University of Cambridge, UK).

2.2.2 Isolation of PBMCs from peripheral human blood

Peripheral blood was diluted with an equal volume of saline, underlayed with an equal volume of Histopaque-1077 and then centrifuged at 1600rpm for 30 mins in a benchtop centrifuge (Beckman, High Wycombe, UK). The PBMC

interface was harvested, washed three times with saline and the number of viable cells counted by trypan blue dye exclusion. The cells were frozen in high grade FCS with 10% DMSO initially at -80°C before transfer to liquid nitrogen for long term storage.

2.2.3 Determination of HLA types of study subjects

2.2.3 (a) Purification of genomic DNA from PBMCs for HLA typing

DNA was purified from frozen PBMCs from subjects of interest using a QIAamp® DNA blood mini kit according to the manufacturer's instructions. Briefly, cells were thawed, washed twice in PBS and counted. $5x10^6$ PBMCs were pelleted in a microfuge tube, then resuspended in 200µl PBS, and 20µl QIAGEN Protease plus 200µl buffer were added. After a 10 minute period of lysis at 56°C, 200µl ethanol was added per tube, mixed and the lysate loaded onto a spin column. The DNA in the sample was bound to the silica-gel membrane of the column during a brief centrifugation step at 6000 x g in a microfuge (Eppendorf, Hamburg, Germany), and the filtrate discarded. Residual contaminants were removed by the addition of two wash buffers, centrifugation and discarding the filtrates. Purified DNA was eluted from the columns by addition of 200µl water and centrifugation for 1 min at 6000 x g. The concentration of DNA in the eluate was determined by measuring the absorbance at 260nm on a GeneQuant Pro UV spectrophotometer (Amersham Pharmacia Biotech, Little Chalfont, UK). Samples were stored at 4°C before sending off for HLA typing.

2.2.3 (b) HLA typing of DNA samples

High resolution class I typing was carried out using 10µg purified DNA by the Oxford Transplant Centre at the Churchill Hospital, Oxford, UK using phototyping (Bunce *et al.*, 1995), a PCR based technique which uses sequence specific primers (PCR-SSP).

2.2.4 Removal of $CD8^+$ cells from human PBMCs

$CD8^+$ or $CD4^+$ T cell subsets were depleted from PBMCs using MACS LD columns. PBMCs were thawed, washed twice in MACS buffer (RPMI 1640 medium supplemented with 2% FCS and 2mM EDTA), and resuspended in 80µl buffer per 10^7 cells. 40µl of MACS CD8 MicroBeads (or CD4 beads as a control) were added per 10^7 cells, mixed and incubated at 4°C for 15 mins. Cells were then washed twice and resuspended in a volume of 2ml of buffer

(up to $5x10^8$ cells in 2ml), ready for application to the columns. MACS LD columns were prepared by placing them onto a MACS magnetic separator and letting 2ml of buffer run through the column. 2ml of cell suspension was then applied to each column and the negative fraction allowed to pass through. The columns were next washed twice with 1ml of buffer, and all negative fractions collected. Cells were passed over a second column to increase the purity of the magnetic separation. The $CD8^+$ (or $CD4^+$) cell depleted fractions were collected and counted before use in assays.

2.2.5 Human IFN-γ Enzyme-linked immunospot (ELISPOT) assay

IFN-γ ELISPOT assays were carried out using a method based on that described by Larsson *et al* (Larsson *et al.*, 1999). Multiscreen 96-well filtration plates were coated with 50µl per well of capture antibody (anti-IFNγ monoclonal antibody (mAb) clone 1-DIK diluted to 5ug/ml in 0.1M sodium bicarbonate buffer, pH9.6) overnight at 4°C. Plates were washed four times with filtered PBS and blocked with 50µl per well of filtered RPMI 1640 medium containing 10% heat inactivated pooled human serum for 1 hour at 37°C in a 5% CO_2 environment. Cells were thawed and washed three times in RPMI 1640 supplemented with 15% high grade FCS, counted and resuspended to a concentration of $2x10^6$c/ml in RPMI with 10% human serum, and 100µl cells were added to each well. If recombinant vaccinia viruses were used to stimulate cells, viruses were added to wells at a multiplicity of infection (m.o.i.) of 2 plaque forming units (pfu) /cell. For consistency, all viruses were diluted to the same titre beforehand and added in an equal volume. If peptides were being used to stimulate cells, they were added in a 20µl volume so that the final concentration was 10^{-5}M for screening assays, or at a range of concentrations for peptide titration assays. For a positive control, 20µl PHA was added to a final concentration of 10µg/ml. For negative control wells, cells received either a recombinant vaccina virus (rVV) expressing β-galactosidase in the rVV-stimulated assay, or medium only in the peptide-stimulated assay. All variables were assayed in duplicate or, where possible, triplicate. Plates were incubated at 37°C in a 5% CO_2 environment for 1 hour, after which 30µl/well filtered FCS (high grade) was added. Plates were incubated at 37°C in a 5% CO_2 environment for two days for rVV-stimulated assays (~45 hrs) or overnight (14-20hrs) for peptide-stimulated assays.

To detect bound IFN-γ, plates were washed four times with filtered PBS containing 0.05% Tween-20 before the addition of 50µl detection antibody (biotin conjugated anti-IFNγ mAb clone 7-B6-1 diluted to a final concentration of 1µg/ml) and incubation for 2 hrs at 37°C/5% CO_2. Plates were washed as before then 100µl per well of anti-biotin alkaline phosphatase (AP) was added (final concentration 1µg/ml) and they were incubated for 1-2hr at room temperature. Plates were washed again and after the final wash, plates were taken apart to wash the back of the filters to remove trapped antibody. AP substrate was prepared by diluting a colour development buffer to a 1x solution in sterile water and adding 100µl AP colour reagents A and B per 10ml of substrate solution required. 100µl substrate was added per well and plates were allowed to develop for 30 mins at room temperature. The reaction was stopped by discarding the substrate and rinsing the filters in tap water. Plates were allowed to dry overnight, then spots were counted using an AID automated image analysis system with AID ELISPOT version 2.3 software (Autoimmun Diagnostika GmbH, Stassberg, Germany).

Results are expressed as the mean (of values from duplicate or triplicate test wells) number of spot forming cells (SFC) per 10^6 PBMCs, and are generally shown with the background values (the mean number of spots counted in the negative control wells) subtracted (specific SFC per 10^6 PBMCs).

2.2.6 Comprehensive HIV $CD8^+$ T cell epitope mapping

A peptide pool matrix-based approach was used to comprehensively characterise the HIV-specific T cell response in thirteen HIV-1 seroconverters (Addo *et al.*, 2003). 310 synthetic peptides spanning the entire HIV-1 clade B consensus sequence (the sequences of which are shown in Table 2.1) were arranged in five different peptide matrix systems (as shown in Table 2.2). The peptides were 20 amino acids in length and overlapped by 10 amino acids. Overlapping peptides corresponding to sequences in Gag, Pol, Nef and Env were arranged as individual peptide matrices while Rev, Tat, Vif, Vpr and Vpu were pooled into a combined accessory and regulatory protein peptide matrix. Within a given protein matrix, each peptide was represented in two different peptide pools, allowing for the identification of an epitope-containing peptide by the patient's cells responding to the two pools containing this peptide.

Peptide number	Matrix assignment	Protein	Sequence
1	gp160 1	gp160 1-20	MRVKGIRKNYQHLWRWGTML
2	gp160 2	gp160 11-30	QHLWRWGTMLLGMLMICSAA
3	gp160 3	gp160 21-40	LGMLMICSAAEQLWVTVYYG
4	gp160 4	gp160 31-50	EQLWVTVYYGVPVWKEATTT
5	gp160 5	gp160 41-60	VPVWKEATTTLFCASDAKAY
6	gp160 6	gp160 51-70	LFCASDAKAYDTEVHNVWAT
7	gp160 7	gp160 61-80	DTEVHNVWATHACVPTDPNP
8	gp160 8	gp160 71-90	HACVPTDPNPQEVVLENVTE
9	gp160 9	gp160 81-100	QEVVLENVTENFNMWKNNMV
10	gp160 10	gp160 91-110	NFNMWKNNMVEQMHEDIISL
11	gp160 11	gp160 101-120	EQMHEDIISLWDQSLKPCVK
12	gp160 12	gp160 111-130	WDQSLKPCVKLTPLCVTLNC
13	gp160 13	gp160 121-140	LTPLCVTLNCTDLNNNTNTT
14	gp160 14	gp160 131-150	TDLNNNTNTTSSSGEKMEKG
15	gp160 15	gp160 141-160	SSSGEKMEKGEIKNCSFNIT
16	gp160 16	gp160 151-170	EIKNCSFNITTSIRDKVQKE
17	gp160 17	gp160 161-180	TSIRDKVQKEYALFYKLDVV
18	gp160 18	gp160 171-190	YALFYKLDVVPIDNDNTSYR
19	gp160 19	gp160 181-200	PIDNDNTSYRLISCNTSVIT
20	gp160 20	gp160 191-210	LISCNTSVITQACPKVSFEP
21	gp160 21	gp160 201-220	QACPKVSFEPIPIHYCAPAG
22	gp160 22	gp160 211-230	IPIHYCAPAGFAILKCNDKK
23	gp160 23	gp160 221-240	FAILKCNDKKFNGTGPCTNV
24	gp160 24	gp160 231-250	FNGTGPCTNVSTVQCTHGIR
25	gp160 25	gp160 241-260	STVQCTHGIRPVVSTQLLLN
26	gp160 26	gp160 251-270	PVVSTQLLLNGSLAEEEVVI
27	gp160 27	gp160 261-280	GSLAEEEVVIRSENFTDNAK
28	gp160 28	gp160 271-290	RSENFTDNAKTIIVQLNESV
29	gp160 29	gp160 281-300	TIIVQLNESVEINCTRPNNN
30	gp160 30	gp160 291-310	EINCTRPNNNTRKSIHIGPG
31	gp160 31	gp160 301-320	TRKSIHIGPGRAFYTTGEII
32	gp160 32	gp160 311-330	RAFYTTGEIIGDIRQAHCNI
33	gp160 33	gp160 321-340	GDIRQAHCNISRAKWNNTLK
34	gp160 34	gp160 331-350	SRAKWNNTLKQIVIKLREQF
35	gp160 35	gp160 341-360	QIVIKLREQFGNKTIVFNQS
36	gp160 36	gp160 351-370	GNKTIVFNQSSGGDPEIVMH
37	gp160 37	gp160 361-380	SGGDPEIVMHSFNCGGEFFY
38	gp160 38	gp160 371-390	SFNCGGEFFYCNTTQLFNST
39	gp160 39	gp160 381-400	CNTTQLFNSTWNSTNTEGSN
40	gp160 40	gp160 391-410	WNSTNTEGSNNTDTITLPCR
41	gp160 41	gp160 401-420	NTDTITLPCRIKQIINMWQE
42	gp160 42	gp160 411-430	IKQIINMWQEVGKAMYAPPI
43	gp160 43	gp160 421-440	VGKAMYAPPIRGQIRCSSNI
44	gp160 44	gp160 431-450	RGQIRCSSNITGLLLTRDGG
45	gp160 45	gp160 441-460	TGLLLTRDGGNNNNTTEIFR
46	gp160 46	gp160 451-470	NNNNTTEIFRPGGGDMRDNW
47	gp160 47	gp160 461-480	PGGGDMRDNWRSELYKYKVV
48	gp160 48	gp160 471-490	RSELYKYKVVKIEPLGVAPT
49	gp160 49	gp160 481-500	KIEPLGVAPTKAKRRVVQRE

50	gp160 50	gp160 491-510	KAKRRVVQREKRAVGIGAMF
51	gp160 51	gp160 501-520	KRAVGIGAMFLGFLGAAGST
52	gp160 52	gp160 511-530	LGFLGAAGSTMGAASMTLTV
53	gp160 53	gp160 521-540	MGAASMTLTVQARQLLSGIV
54	gp160 54	gp160 531-550	QARQLLSGIVQQQNNLLRAI
55	gp160 55	gp160 541-560	QQQNNLLRAIEAQQHLLQLT
56	gp160 56	gp160 551-570	EAQQHLLQLTVWGIKQLQAR
57	gp160 57	gp160 561-580	VWGIKQLQARVLAVERYLKD
58	gp160 58	gp160 571-590	VLAVERYLKDQQLLGIWGCS
59	gp160 59	gp160 581-600	QQLLGIWGCSGKLICTTAVP
60	gp160 60	gp160 591-610	GKLICTTAVPWNASWSNKSL
61	gp160 61	gp160 601-620	WNASWSNKSLDQIWNNMTWM
62	gp160 62	gp160 611-630	DQIWNNMTWMEWEREIDNYT
63	gp160 63	gp160 621-640	EWEREIDNYTSLIYTLIEES
64	gp160 64	gp160 631-650	SLIYTLIEESQNQQEKNEQE
65	gp160 65	gp160 641-660	QNQQEKNEQELLELDKWASL
66	gp160 66	gp160 651-670	LLELDKWASLWNWFDITNWL
67	gp160 67	gp160 661-680	WNWFDITNWLWYIKIFIMIV
68	gp160 68	gp160 671-690	WYIKIFIMIVGGLVGLRIVF
69	gp160 69	gp160 681-700	GGLVGLRIVFAVLSIVNRVR
70	gp160 70	gp160 691-710	AVLSIVNRVRQGYSPLSFQT
71	gp160 71	gp160 701-720	QGYSPLSFQTRLPAPRGPDR
72	gp160 72	gp160 711-730	RLPAPRGPDRPEGIEEEGGE
73	gp160 73	gp160 721-740	PEGIEEEGGERDRDRSGRLV
74	gp160 74	gp160 731-750	RDRDRSGRLVDGFLALIWVD
75	gp160 75	gp160 741-760	DGFLALIWVDLRSLCLFSYH
76	gp160 76	gp160 751-770	LRSLCLFSYHRLRDLLLIVT
77	gp160 77	gp160 761-780	RLRDLLLIVTRIVELLGRRG
78	gp160 78	gp160 771-790	RIVELLGRRGWEALKYWWNL
79	gp160 79	gp160 781-800	WEALKYWWNLLQYWSQELKN
80	gp160 80	gp160 791-810	LQYWSQELKNSAVSLLNATA
81	gp160 81	gp160 801-820	SAVSLLNATAIAVAEGTDRV
82	gp160 82	gp160 811-830	IAVAEGTDRVIEVVQRACRA
83	gp160 83	gp160 821-840	IEVVQRACRAILHIPRRIRQ
84	gp160 84	gp160 828-847	CRAILHIPRRIRQGLERALL
85	gag 1	gag p17 1-20	MGARASVLSGGELDRWEKIR
86	gag 2	gag p17 11-30	GELDRWEKIRLRPGGKKKYK
87	gag 3	gag p17 21-40	LRPGGKKKYKLKHIVWASRE
88	gag 4	gag p17 31-50	LKHIVWASRELERFAVNPGL
89	gag 5	gag p17 41-60	LERFAVNPGLLETSEGCRQI
90	gag 6	gag p17 51-70	LETSEGCRQILGQLQPSLQT
91	gag 7	gag p17 61-80	LGQLQPSLQTGSEELRSLYN
92	gag 8	gag p17 71-90	GSEELRSLYNTVATLYCVHQ
93	gag 9	gag p17 81-100	TVATLYCVHQRIEVKDTKEA
94	gag 10	gag p17 91-110	RIEVKDTKEALEKIEEEQNK
95	gag 11	gag p17 101-120	LEKIEEEQNKSKKKAQQAAA
96	gag 12	gag p17 111-130	SKKKAQQAAADTGNSSQVSQ
97	gag 13	gag p17 121-132/gag p24 1-8	DTGNSSQVSQNYPIVQNLQG
98	gag 14	gag p17 131-132/gag p24 1-18	NYPIVQNLQGQMVHQAISPR
99	gag 15	gag p24 9-28	QMVHQAISPRTLNAWVKVVE

100	gag 16	gag p24 19-38	TLNAWVKVVEEKAFSPEVIP
101	gag 17	gag p24 29-48	EKAFSPEVIPMFSALSEGAT
102	gag 18	gag p24 39-58	MFSALSEGATPQDLNTMLNT
103	gag 19	gag p24 49-68	PQDLNTMLNTVGGHQAAMQM
104	gag 20	gag p24 59-78	VGGHQAAMQMLKETINEEAA
105	gag 21	gag p24 69-88	LKETINEEAAEWDRLHPVHA
106	gag 22	gag p24 79-98	EWDRLHPVHAGPIAPGQMRE
107	gag 23	gag p24 89-108	GPIAPGQMREPRGSDIAGTT
108	gag 24	gag p24 99-118	PRGSDIAGTTSTLQEQIGWM
109	gag 25	gag p24 109-128	STLQEQIGWMTNNPPIPVGE
110	gag 26	gag p24 119-138	TNNPPIPVGEIYKRWIILGL
111	gag 27	gag p24 129-148	IYKRWIILGLNKIVRMYSPT
112	gag 28	gag p24 139-158	NKIVRMYSPTSILDIRQGPK
113	gag 29	gag p24 149-168	SILDIRQGPKEPFRDYVDRF
114	gag 30	gag p24 159-178	EPFRDYVDRFYKTLRAEQAS
115	gag 31	gag p24 169-188	YKTLRAEQASQEVKNWMTET
116	gag 32	gag p24 179-198	QEVKNWMTETLLVQNANPDC
117	gag 33	gag p24 189-208	LLVQNANPDCKTILKALGPA
118	gag 34	gag p24 199-218	KTILKALGPAATLEEMMTAC
119	gag 35	gag p24 209-228	ATLEEMMTACQGVGGPGHKA
120	gag 36	gag p24 219-231/gag p2p7p6p1 1-7	QGVGGPGHKARVLAEAMSQV
121	gag 37	gag p24 229-231/gag p2p7p6p1 1-17	RVLAEAMSQVTNSATIMMQR
122	gag 38	gag p2p7p1p6 8-27	TNSATIMMQRGNFRNQRKTV
123	gag 39	gag p2p7p1p6 18-37	GNFRNQRKTVKCFNCGKEGH
124	gag 40	gag p2p7p1p6 28-47	KCFNCGKEGHIAKNCRAPRK
125	gag 41	gag p2p7p1p6 38-57	IAKNCRAPRKKGCWKCGKEG
126	gag 42	gag p2p7p1p6 48-67	KGCWKCGKEGHQMKDCTERQ
127	gag 43	gag p2p7p1p6 58-77	HQMKDCTERQANFLGKIWPS
128	gag 44	gag p2p7p1p6 68-87	ANFLGKIWPSHKGRPGNFLQ
129	gag 45	gag p2p7p1p6 78-97	HKGRPGNFLQSRPEPTAPPE
130	gag 46	gag p2p7p1p6 88-107	SRPEPTAPPEESFRFGEETT
131	gag 47	gag p2p7p1p6 98-117	ESFRFGEETTTPSQKQEPID
132	gag 48	gag p2p7p1p6 108-127	TPSQKQEPIDKELYPLASLR
133	gag 49	gag p2p7p1p6 118-137	KELYPLASLRSLFGNDPSSQ
134	pol 1	pol	FFREDLAFPQGKAREFSSEQ
135	pol 2	pol	GKAREFSSEQTRANSPTRRE
136	pol 3	pol	TRANSPTRRELQVWGRDNNS
137	pol 4	pol	LQVWGRDNNSLSEAGADRQG
138	pol 5	pol	LSEAGADRQGTVSFSFPQIT
139	pol 6	pol	TVSFSFPQITLWQRPLVTIK
140	pol 7	protease 5-24	LWQRPLVTIKIGGQLKEALL
141	pol 8	protease 15-34	IGGQLKEALLDTGADDTVLE
142	pol 9	protease 25-44	DTGADDTVLEEMNLPGRWKP
143	pol 10	protease 35-54	EMNLPGRWKPKMIGGIGGFI
144	pol 11	protease 45-64	KMIGGIGGFIKVRQYDQILI
145	pol 12	protease 55-74	KVRQYDQILIEICGHKAIGT
146	pol 13	protease 65-84	EICGHKAIGTVLVGPTPVNI
147	pol 14	protease 75-94	VLVGPTPVNIIGRNLLTQIG
148	pol 15	protease 85-99/RT 1-5	IGRNLLTQIGCTLNFPISPI
149	pol 16	protease 95-99/RT 1-15	CTLNFPISPIETVPVKLKPG

150	pol 17	RT 6-25	ETVPVKLKPGMDGPKVKQWP
151	pol 18	RT 16-35	MDGPKVKQWPLTEEKIKALV
152	pol 19	RT 26-45	LTEEKIKALVEICTEMEKEG
153	pol 20	RT 36-55	EICTEMEKEGKISKIGPENP
154	pol 21	RT 46-65	KISKIGPENPYNTPVFAIKK
155	pol 22	RT 56-75	YNTPVFAIKKKDSTKWRKLV
156	pol 23	RT 66-85	KDSTKWRKLVDFRELNKRTQ
157	pol 24	RT 76-95	DFRELNKRTQDFWEVQLGIP
158	pol 25	RT 86-105	DFWEVQLGIPHPAGLKKKKS
159	pol 26	RT 96-115	HPAGLKKKKSVTVLDVGDAY
160	pol 27	RT 106-125	VTVLDVGDAYFSVPLDKDFR
161	pol 28	RT 116-135	FSVPLDKDFRKYTAFTIPSI
162	pol 29	RT 126-145	KYTAFTIPSINNETPGIRYQ
163	pol 30	RT 136-155	NNETPGIRYQYNVLPQGWKG
164	pol 31	RT 146-165	YNVLPQGWKGSPAIFQSSMT
165	pol 32	RT 156-175	SPAIFQSSMTKILEPFRKQN
166	pol 33	RT 166-185	KILEPFRKQNPDIVIYQYMD
167	pol 34	RT 176-195	PDIVIYQYMDDLYVGSDLEI
168	pol 35	RT 186-205	DLYVGSDLEIGQHRTKIEEL
169	pol 36	RT 196-215	GQHRTKIEELRQHLLRWGFT
170	pol 37	RT 206-225	RQHLLRWGFTTPDKKHQKEP
171	pol 38	RT 216-235	TPDKKHQKEPPFLWMGYELH
172	pol 39	RT 226-245	PFLWMGYELHPDKWTVQPIV
173	pol 40	RT 236-255	PDKWTVQPIVLPEKDSWTVN
174	pol 41	RT 246-265	LPEKDSWTVNDIQKLVGKLN
175	pol 42	RT 256-275	DIQKLVGKLNWASQIYAGIK
176	pol 43	RT 266-285	WASQIYAGIKVKQLCKLLRG
177	pol 44	RT 276-295	VKQLCKLLRGTKALTEVIPL
178	pol 45	RT286-305	TKALTEVIPLTEEAELELAE
179	pol 46	RT 296-315	TEEAELELAENREILKEPVH
180	pol 47	RT 306-325	NREILKEPVHGVYYDPSKDL
181	pol 48	RT 316-335	GVYYDPSKDLIAEIQKQGQG
182	pol 49	RT 326-345	IAEIQKQGQGQWTYQIYQEP
183	pol 50	RT 336-355	QWTYQIYQEPFKNLKTGKYA
184	pol 51	RT 346-365	FKNLKTGKYARMRGAHTNDV
185	pol 52	RT 356-375	RMRGAHTNDVKQLTEAVQKI
186	pol 53	RT 366-385	KQLTEAVQKIATESIVIWGK
187	pol 54	RT 376-395	ATESIVIWGKTPKFKLPIQK
188	pol 55	RT 386-405	TPKFKLPIQKETWEAWWTEY
189	pol 56	RT 396-415	ETWEAWWTEYWQATWIPEWE
190	pol 57	RT 406-425	WQATWIPEWEFVNTPPLVKL
191	pol 58	RT 416-435	FVNTPPLVKLWYQLEKEPIV
192	pol 59	RT 426-445	WYQLEKEPIVGAETFYVDGA
193	pol 60	RT 436-455	GAETFYVDGAANRETKLGKA
194	pol 61	RT 446-465	ANRETKLGKAGYVTDRGRQK
195	pol 62	RT 456-475	GYVTDRGRQKVVSLTDTTNQ
196	pol 63	RT 466-485	VVSLTDTTNQKTELQAIHLA
197	pol 64	RT 476-495	KTELQAIHLALQDSGLEVNI
198	pol 65	RT486-505	LQDSGLEVNIVTDSQYALGI
199	pol 66	RT 496-515	VTDSQYALGIIQAQPDKSES

200	pol 67	RT 506-525	IQAQPDKSESELVSQIIEQL
201	pol 68	RT 516-535	ELVSQIIEQLIKKEKVYLAW
202	pol 69	RT 526-545	IKKEKVYLAWVPAHKGIGGN
203	pol 70	RT 536-555	VPAHKGIGGNEQVDKLVSAG
204	pol 71	RT 546-560/integrase 1-5	EQVDKLVSAGIRKVLFLDGI
205	pol 72	RT 556-560/integrase 1-15	IRKVLFLDGIDKAQEEHEKY
206	pol 73	integrase 6-25	DKAQEEHEKYHSNWRAMASD
207	pol 74	integrase 16-35	HSNWRAMASDFNLPPVVAKE
208	pol 75	integrase 26-45	FNLPPVVAKEIVASCDKCQL
209	pol 76	integrase 36-55	IVASCDKCQLKGEAMHGQVD
210	pol 77	integrase 46-65	KGEAMHGQVDCSPGIWQLDC
211	pol 78	integrase 56-75	CSPGIWQLDCTHLEGKIILV
212	pol 79	integrase 66-85	THLEGKIILVAVHVASGYIE
213	pol 80	integrase 76-95	AVHVASGYIEAEVIPAETGQ
214	pol 81	integrase 86-105	AEVIPAETGQETAYFLLKLA
215	pol 82	integrase 96-115	ETAYFLLKLAGRWPVKTIHT
216	pol 83	integrase 106-125	GRWPVKTIHTDNGSNFTSTT
217	pol 84	integrase 116-135	DNGSNFTSTTVKAACWWAGI
218	pol 85	integrase 126-145	VKAACWWAGIKQEFGIPYNP
219	pol 86	integrase 136-155	KQEFGIPYNPQSQGVVESMN
220	pol 87	integrase 146-165	QSQGVVESMNKELKKIIGQV
221	pol 88	integrase 156-175	KELKKIIGQVRDQAEHLKTA
222	pol 89	integrase 166-185	RDQAEHLKTAVQMAVFIHNF
223	pol 90	integrase 176-195	VQMAVFIHNFKRKGGIGGYS
224	pol 91	integrase 186-205	KRKGGIGGYSAGERIVDIIA
225	pol 92	integrase 196-215	AGERIVDIIATDIQTKELQK
226	pol 93	integrase 206-225	TDIQTKELQKQITKIQNFRV
227	pol 94	integrase 216-235	QITKIQNFRVYYRDSRDPLW
228	pol 95	integrase 226-245	YYRDSRDPLWKGPAKLLWKG
229	pol 96	integrase 236-255	KGPAKLLWKGEGAVVIQDNS
230	pol 97	integrase 246-265	EGAVVIQDNSDIKVVPRRKA
231	pol 98	integrase 256-275	DIKVVPRRKAKIIRDYGKQM
232	pol 99	integrase 266-285	KIIRDYGKQMAGDDCVASRQ
233	pol 100	integrase 269-288	RDYGKQMAGDDCVASRQDED
234	nef 1	nef 1-20	MGGKWSKRSVVGWPTVRERM
235	nef 2	nef 11-30	VGWPTVRERMRRAEPAADGV
236	nef 3	nef 21-40	RRAEPAADGVGAVSRDLEKH
237	nef 4	nef 31-50	GAVSRDLEKHGAITSSNTAA
238	nef 5	nef 41-60	GAITSSNTAANNADCAWLEA
239	nef 6	nef 51-70	NNADCAWLEAQEEEEVGFPV
240	nef 7	nef 61-80	QEEEEVGFPVRPQVPLRPMT
241	nef 8	nef 71-90	RPQVPLRPMTYKAAVDLSHF
242	nef 9	nef 81-100	YKAAVDLSHFLKEKGGLEGL
243	nef 10	nef 91-110	LKEKGGLEGLIYSQKRQDIL
244	nef 11	nef 101-120	IYSQKRQDILDLWVYHTQGY
245	nef 12	nef 111-130	DLWVYHTQGYFPDWQNYTPG
246	nef 13	nef 121-140	FPDWQNYTPGPGIRYPLTFG
247	nef 14	nef 131-150	PGIRYPLTFGWCFKLVPVEP
248	nef 15	nef 141-160	WCFKLVPVEPEKVEEANEGE
249	nef 16	nef 151-170	EKVEEANEGENNSLLHPMSL

250	nef 17	nef 161-180	NNSLLHPMSLHGMDDPEREV
251	nef 18	nef 171-190	HGMDDPEREVLVWKFDSRLA
252	nef 19	nef 181-200	LVWKFDSRLAFHHMARELHP
253	nef 20	nef 187-206	SRLAFHHMARELHPEYYKDC
254	rev 1	rev 1-20	MAGRSGDSDEELLKTVRLIK
255	rev 2	rev 11-30	ELLKTVRLIKFLYQSNPPPS
256	rev 3	rev 21-40	FLYQSNPPPSPEGTRQARRN
257	rev 4	rev 31-50	PEGTRQARRNRRRRWRERQR
258	rev 5	rev 41-60	RRRRWRERQRQIRSISEWIL
259	rev 6	rev 51-70	QIRSISEWILSTYLGRPAEP
260	rev 7	rev 61-80	STYLGRPAEPVPLQLPPLER
261	rev 8	rev 71-90	VPLQLPPLERLTLDCNEDCG
262	rev 9	rev 81-100	LTLDCNEDCGTSGTQGVGSP
263	rev 10	rev 91-110	TSGTQGVGSPQILVESPAVL
264	rev 11	rev 97-116	VGSPQILVESPAVLESGTKE
265	tat 1	tat 1-20	MEPVDPRLEPWKHPGSQPKT
266	tat 2	tat 11-30	WKHPGSQPKTACTNCYCKKC
267	tat 3	tat 21-40	ACTNCYCKKCCFHCQVCFIT
268	tat 4	tat 31-50	CFHCQVCFITKGLGISYGRK
269	tat 5	tat 41-60	KGLGISYGRKKRRQRRRAPQ
270	tat 6	tat 51-70	KRRQRRRAPQDSQTHQVSLS
271	tat 7	tat 61-80	DSQTHQVSLSKQPASQPRGD
272	tat 8	tat 71-90	KQPASQPRGDPTGPKESKKK
273	tat 9	tat 81-100	PTGPKESKKKVERETETDPV
274	tat 10	tat 82-101	TGPKESKKKVERETETDPVD
275	vif 1	vif 1-20	MENRWQVMIVWQVDRMRIRT
276	vif 2	vif 11-30	WQVDRMRIRTWKSLVKHHMY
277	vif 3	vif 21-40	WKSLVKHHMYISRKAKGWFY
278	vif 4	vif 31-50	ISRKAKGWFYRHHYESTHPR
279	vif 5	vif 41-60	RHHYESTHPRISSEVHIPLG
280	vif 6	vif 51-70	ISSEVHIPLGDARLVITTYW
281	vif 7	vif 61-80	DARLVITTYWGLHTGERDWH
282	vif 8	vif 71-90	GLHTGERDWHLGQGVSIEWR
283	vif 9	vif 81-100	LGQGVSIEWRKKRYSTQVDP
284	vif 10	vif 91-110	KKRYSTQVDPDLADQLIHLY
285	vif 11	vif 101-120	DLADQLIHLYYFDCFSESAI
286	vif 12	vif 111-130	YFDCFSESAIRNAILGHIVS
287	vif 13	vif 121-140	RNAILGHIVSPRCEYQAGHN
288	vif 14	vif 131-150	PRCEYQAGHNKVGSLQYLAL
289	vif 15	vif 141-160	KVGSLQYLALAALITPKKIK
290	vif 16	vif 151-170	AALITPKKIKPPLPSVTKLT
291	vif 17	vif 161-180	PPLPSVTKLTEDRWNKPQKT
292	vif 18	vif 171-190	EDRWNKPQKTKGHRGSHTMN
293	vif 19	vif 173-192	RWNKPQKTKGHRGSHTMNGH
294	vpr 1	vpr 1-20	MEQAPEDQGPQREPYNEWTL
295	vpr 2	vpr 11-30	QREPYNEWTLELLEELKSEA
296	vpr 3	vpr 21-40	ELLEELKSEAVRHFPRIWLH
297	vpr 4	vpr 31-50	VRHFPRIWLHGLGQHIYETY
298	vpr 5	vpr 41-60	GLGQHIYETYGDTWAGVEAI
299	vpr 6	vpr 51-70	GDTWAGVEAIIRILQQLLFI

300	vpr 7	vpr 61-80	IRILQQLLFIHFRIGCQHSR
301	vpr 8	vpr 71-90	HFRIGCQHSRIGITRQRRAR
302	vpr 9	vpr 77-96	QHSRIGITRQRRARNGASRS
303	vpu 1	vpu 1-20	MQSLQILAIVALVVAAIIAI
304	vpu 2	vpu 11-30	ALVVAAIIAIVVWSIVFIEY
305	vpu 3	vpu 21-40	VVWSIVFIEYRKILRQRKID
306	vpu 4	vpu 31-50	RKILRQRKIDRLIDRIRERA
307	vpu 5	vpu 41-60	RLIDRIRERAEDSGNESEGD
308	vpu 6	vpu 51-70	EDSGNESEGDQEELSALVEM
309	vpu 7	vpu 61-80	QEELSALVEMGHHAPWDVDD
310	vpu 8	vpu 62-81	EELSALVEMGHHAPWDVDDL

Table 2.1. Sequences of long peptides spanning the entire HIV-1 clade B consensus sequence used for comprehensive mapping of HIV epitopes. The sequences of all 310 overlapping peptides (based on a recent clade B consensus sequence http://www.hiv.lanl.gov/content/hiv-db/CONSENSUS/M_GROUP/2002-Aug.html), their designation in the peptide pool matrices in Table 2.2 and the protein/amino acid position within the HIV-1 genome they correspond to are shown.

Matrix setup for gp160 peptides:

	Pool A	Pool B	Pool C	Pool D	Pool E	Pool F	Pool G	Pool H	Pool I	Pool J
Pool K	gp160-1	gp160-2	gp160-3	gp160-4	gp160-5	gp160-6	gp160-7	gp160-8	gp160-9	gp160-10
Pool L	gp160-11	gp160-12	gp160-13	gp160-14	gp160-15	gp160-16	gp160-17	gp160-18	gp160-19	gp160-20
Pool M	gp160-21	gp160-22	gp160-23	gp160-24	gp160-25	gp160-26	gp160-27	gp160-28	gp160-29	gp160-30
Pool N	gp160-31	gp160-32	gp160-33	gp160-34	gp160-35	gp160-36	gp160-37	gp160-38	gp160-39	gp160-40
Pool O	gp160-41	gp160-42	gp160-43	gp160-44	gp160-45	gp160-46	gp160-47	gp160-48	gp160-49	gp160-50
Pool P	gp160-51	gp160-52	gp160-53	gp160-54	gp160-55	gp160-56	gp160-57	gp160-58	gp160-59	gp160-60
Pool Q	gp160-61	gp160-62	gp160-63	gp160-64	gp160-65	gp160-66	gp160-67	gp160-68	gp160-69	gp160-70
Pool R	gp160-71	gp160-72	gp160-73	gp160-74	gp160-75	gp160-76	gp160-77	gp160-78	gp160-79	gp160-80
Pool S	gp160-81	gp160-82	gp160-83	gp160-84						

Matrix setup for gag peptides:

	Pool A	Pool B	Pool C	Pool D	Pool E	Pool F	Pool G
Pool H	gag-1	gag-2	gag-3	gag-4	gag-5	gag-6	gag-7
Pool I	gag-8	gag-9	gag-10	gag-11	gag-12	gag-13	gag-14
Pool J	gag-15	gag-16	gag-17	gag-18	gag-19	gag-20	gag-21
Pool K	gag-22	gag-23	gag-24	gag-25	gag-26	gag-27	gag-28
Pool L	gag-29	gag-30	gag-31	gag-32	gag-33	gag-34	gag-35
Pool M	gag-36	gag-37	gag-38	gag-39	gag-40	gag-41	gag-42
Pool N	gag-43	gag-44	gag-45	gag-46	gag-47	gag-48	gag-49

Matrix setup for pol peptides:

	Pool A	Pool B	Pool C	Pool D	Pool E	Pool F	Pool G	Pool H	Pool I	Pool J
Pool K	pol-1	pol-2	pol-3	pol-4	pol-5	pol-6	pol-7	pol-8	pol-9	pol-10
Pool L	pol-11	pol-12	pol-13	pol-14	pol-15	pol-16	pol-17	pol-18	pol-19	pol-20
Pool M	pol-21	pol-22	pol-23	pol-24	pol-25	pol-26	pol-27	pol-28	pol-29	pol-30
Pool N	pol-31	pol-32	pol-33	pol-34	pol-35	pol-36	pol-37	pol-38	pol-39	pol-40
Pool O	pol-41	pol-42	pol-43	pol-44	pol-45	pol-46	pol-47	pol-48	pol-49	pol-50
Pool P	pol-51	pol-52	pol-53	pol-54	pol-55	pol-56	pol-57	pol-58	pol-59	pol-60
Pool Q	pol-61	pol-62	pol-63	pol-65	pol-65	pol-66	pol-67	pol-68	pol-69	pol-70
Pool R	pol-71	pol-72	pol-73	pol-74	pol-75	pol-76	pol-77	pol-78	pol-79	pol-80
Pool S	pol-81	pol-82	pol-83	pol-84	pol-85	pol-86	pol-87	pol-88	pol-89	pol- 90
Pool T	pol-91	pol-92	pol-93	pol-94	pol-95	pol-96	pol-97	pol-98	pol-99	pol-100

Matrix setup for nef peptides:

	Pool A	Pool B	Pool C	Pool D
Pool E	nef-1	nef-2	nef-3	nef-4
Pool F	nef-5	nef-6	nef-7	nef-8
Pool G	nef-9	nef-10	nef-11	nef-12
Pool H	nef-13	nef-14	nef-15	nef-16
Pool I	nef-17	nef-18	nef-19	nef-20

Matrix setup for accessory and regulatory proteins:

	Pool A	Pool B	Pool C	Pool D	Pool E	Pool F	Pool G	Pool H
Pool I	rev-1	rev-2	rev-3	rev-4	rev-5	rev-6	rev-7	rev-8
Pool J	rev-9	rev-10	rev-11	tat-1	tat-2	tat-3	tat-4	tat-5
Pool K	tat-6	tat-7	tat-8	tat-9	tat-10	vif-1	vif-2	vif-3
Pool L	vif-4	vif-5	vif-6	vif-7	vif-8	vif-9	vif-10	vif-11
Pool M	vif-12	vif-13	vif-14	vif-15	vif-16	vif-17	vif-18	vif-19
Pool N	vpr-1	vpr-2	vpr-3	vpr-4	vpr-5	vpr-6	vpr-7	vpr-8
Pool O	vpr-9	vpu-1	vpu-2	vpu-3	vpu-4	vpu-5	vpu-6	vpu-7
Pool P	vpu-8							

Table 2.2. Arrangement of peptides corresponding to the entire HIV-1 clade B consensus sequence into peptide pools according to a matrix system. A panel of 310 overlapping peptides, 20 amino acids in length overlapping by 10 amino acids, was arranged into 78 peptide pools as shown in the matrices above, where each peptide is represented in two separate pools. Peptides spanning HIV gp160, gag, pol and nef were combined into separate individual matrices, and peptides spanning rev, tat, vif, vpr and vpu were arranged in a combined accessory/regulatory protein matrix. Each peptide was assigned a peptide number, for example the 84 peptides spanning gp160 were named gp160-1 to gp160-84. The amino acid sequences of each peptide are given in Table 2.1.

Cryopreserved patient PBMCs were screened for responses to all peptide pools by IFN-γ ELISPOT assay. To enable determination of specific responses over background levels of IFN-γ production, control wells containing cells stimulated with medium only were included on each plate. From the matrices, a ranked list of potential epitope-containing peptides was made (in accordance with which peptide pools stimulated the largest responses), and the top 25-30 peptides most likely to contain an epitope were re-tested individually in a second IFN-γ ELISPOT assay. In the second assay to confirm which peptides corresponded to epitopic regions, only those which stimulated a response which was greater than three times a background value (the mean of the values of the IFN-γ spot numbers counted in the wells containing cells stimulated with medium alone) and at least 50 SFC per 10^6 PBMC were considered to be genuine, positive responses.

The number of epitopic regions recognised, and the relative magnitude of the response to each region were calculated for each patient. In order to avoid overestimation of the number of epitopes recognised, responses to two adjacent overlapping peptides were counted as a response to one epitope, since some epitopes could be located in the overlapping region of two adjacent peptides, resulting in responses to both overlapping peptides. Only the higher of the responses observed to two adjacent overlapping peptides were considered for final calculations of the overall magnitude of the HIV-specific T cell response.

2.2.7 Statistical analysis

Statistical analysis of data was performed using Graphpad Prism version 4.00 software. Pearson's correlation test was used to estimate the degree of association between the concurrent viral load and the different parameters of the early HIV-specific $CD8^+$ T cell response studied in chapter 3; between the breadth and magnitude of total HIV-specific $CD8^+$ T cell responses; between the functional avidity and relative dominance of HIV epitope-specific responses; and between the kinetics of expansion and magnitude of HIV epitope-specific responses. Linear regression analysis was used to fit a line through data points plotted on scatter graphs and determine the slope.

Two-tailed t-tests were used to assess whether the differences between the means of groups of observations (e.g. antigen-specific responses made by

animals in different groups or the functional avidities of responses of different dominance) were statistically significant.

One-way analysis of variance (ANOVA) was used to compare the means of groups of observations recorded in three or more groups of subjects (e.g. features of the HIV-specific response made in patient who established different persisting viral loads, or antigen-specific responses made by mice receiving different treatments) to see if the difference across the groups was significant. Tukey's multiple comparison tests were subsequently carried out to compare pairs of group means.

2.2.8 Staining human PBMCs with antibodies to cell surface markers and tetramers (or pentamers)

In 96-well plates, $5x10^5$-10^6 PBMCs per well were stained with a tetramer or pentamer in a 50µl volume and incubated according to pre-optimised conditions. (Each tetramer or pentamer had been tested on cells from a patient known to have a response to the epitope plus a HLA-matched HIV seronegative donor as a control using different concentrations, temperatures and incubation times to determine the minimum concentration and combination of conditions which gave the brightest specific staining and lowest background staining.) Cells were washed three times in PBS supplemented with 10% FCS, and then potential Fc receptor-mediated binding of antibodies blocked by incubating the cells for 15 mins at 4°C in PBS supplemented with 10% FCS and 10% human serum. Cells were pelleted and relevant monoclonal antibodies (mAbs) to cell surface markers added in a 50µl volume and incubated for 15 mins at 4°C. Cells were then washed three times and either fixed using 200µl 4% paraformaldehyde or further staining (and blocking if necessary) steps performed. Some samples were stained with a single antibody only (without staining with a tetramer); for these samples, a pre-blocking step with FCS was not necessary. The proportion of cells expressing the marker(s) of interest was determined by fluorescence-activated cell sorting (FACS): samples were run on a FACS Calibur (Beckton Dickinson, Oxford, UK) and data was analysed using Cell Quest Pro software.

2.2.9 Staining human PBMCs with antibodies against intracellular markers

Cell were permeabilised by incubating with 100µl BD cytofix/perm solution for 20 mins at 4°C, followed by three washes with BD perm/wash buffer. Potential FcR-mediated binding of antibodies was prevented by blocking with

perm/wash buffer containing 10% human serum. Antibodies to intracellular markers were added to pelleted cells and incubated for 15 mins at 4°C. Cells were washed three times in perm/wash buffer and then fixed using 4% paraformaldehyde. Samples were run on a FACS Calibur and data was analysed using Cell Quest Pro software.

2.2.10 Analysis of Vβ family usage by HIV-specific $CD8^+$ cells

Vβ family usage by HIV-specific $CD8^+$ cells was assessed by co-staining tetramer- or pentamer-labelled cells with Vβ family-specific antibodies (either, as indicated in the materials, directly conjugated to a flurorchrome or detected using secondary reagents). Staining was carried out by the same method employed for analysis of cell surface markers on HIV-specific $CD8^+$ T cells, described in section 2.2.8. The percentage of tetramer-stained cells utilising each Vβ family was subsequently determined by FACS analysis, using Cell Quest Pro software.

2.2.11 Stimulation and expansion of polyclonal CTL from HIV-infected individuals

Frozen PBMCs were thawed quickly at 37°C and then washed once in T cell growth medium (RPMI 1640 supplemented with 10% high grade FCS, 50U/ml penicillin, 50µg/ml streptomycin, 10U/ml IL-2 and 50µM 2-mercaptoethanol). Cells were counted and ~4-5x10^5 PBMCs were plated in a 1ml volume into wells of a 24 well plate. To each well, 1ml of medium containing 5x10^5 irradiated allogeneic feeder cells and 0.8µg/ml anti-CD3 antibody was added. Cells were incubated at 37°C for 8 days with changes in the medium made every 3 days. The resulting polyclonal T cells were assayed for HIV-specific CTL activity using a ^{51}Cr release assay.

2.2.12 ^{51}Cr release assay

^{51}Cr release assays were performed as described by Borrow *et al* (Borrow *et al.*, 1994). Autologous EBV-B-LCL (EBV-transformed B lymphoblastoid cell line) to be used as target cells were counted and 2x10^6c pelleted and labelled with 100µCi $NaCrO_4$ for 1 hour at 37°C. ^{51}Cr-labelled cells were then washed four times in CTL assay medium (RPMI without phenol red supplemented with 10% high grade FCS), and then resuspended at 3x10^5c/ml and added to assay wells in 50µl (1.5x10^4 target cells/well). Effector CTL were harvested from 24 well plates, pelleted and resuspended in CTL assay medium at an

appropriate concentration so that 100µl could be added to test wells to give an effector:target (E:T) ratio of at least 50:1. To assay the recognition of synthetic peptides, peptide was added in 50µl at 4x the desired concentration. Controls were included to indicate the spontaneous release of ^{51}Cr from the target cells (measured by adding target cells and medium alone in a total volume of 200µl/well) and the maximal lysis (measured by adding target cells in a 100µl volume and 100µl of 2% (v/v) Triton-X-100 per well). All variables were assayed in triplicate.

Assays were incubated at 37°C for 5 hours. 40µl of supernatant was then harvested from each well into a counting plate and 100µl scintillant (Opti-phase supermix) was added. The plates were sealed with adhesive lids and left overnight for the scintillant and sample to become homogeneously mixed before they were then counted on a Beta-plate reader (Microbeta Trilux, Wallac, Turku, Finland). The percent specific ^{51}Cr release was calculated as detailed below:

$$\frac{\text{(mean test counts - mean spontaneous counts)}}{\text{(mean maximum counts - mean spontaneous counts)}} \times 100 = \%\ \text{specific}\ ^{51}Cr\ \text{release}$$

2.2.13 Assay to determine the relative affinity of binding of peptides to MHC alleles

The relative affinity of binding of peptides to murine H-2 alleles was determined using a MHC stabilisation assay (Gairin *et al.*, 1995). Test peptides were serially diluted in AIM V medium across 96 well plates from an initial starting concentration of 2mM, down to 0.2nM. Peptides were prepared in a 100µl volume at twice the desired final concentration as they were to be diluted by the addition of an equal volume of cells. Dilutions of positive reference (LCMV NP396 for D^b assay; 2c Syng for K^b assay) and negative control (HIV Nef 82-90) peptides were also prepared in the same way. A no peptide control was also included.

RMA-S cells (endogenously expressing K^b and D^b molecules) previously cultured overnight at 26°C to induce stable MHC expression on the cell surface were washed, counted and $2.5x10^6$ cells in a 100µl volume added to all wells. The cells were incubated with the peptides for one hour at 26°C to allow stable peptide-MHC complexes to form, and then incubated for 3 hrs at 37°C, a temperature at which, "empty" MHC-β_2m complexes dissociate and

disappear from the cell surface. The level of surface MHC expression (and hence the relative affinity of binding of the stabilising peptide) was then assessed by staining the cells with FITC-conjugated anti-class I H-$2K^b$ or H-$2D^b$ antibody (or isotype control antibodies) for one hour at 4°C. Cells were washed twice in FACS buffer and fixed with 4% paraformaldehyde, after which they were stored at 4°C in the dark before running the samples on a FACS Calibur.

The fluorescence intensity of each sample was analysed using CellQuest software. Results are expressed as fluorescence index (FI) values. These were calculated according to the following formula:

$$\frac{\text{mean fluorescence intensity (MFI) of test peptide - MFI of no peptide isotype control}}{\text{MFI of no peptide class I-stained control – MFI of no peptide isotype control}}$$

The half-maximal binding level (BL_{50}), which is the peptide concentration yielding the half-maximal FI of the reference peptide in each assay, was calculated using ED50 plus v1.0 software.

2.2.14 Propagation of viruses

2.2.14 (a) Growth of working stocks

- Recombinant vaccinia viruses

143 TK^- cells were set up in flasks at 10^7 cells/flask in a volume of ~30ml medium (Eagle's MEM supplemented with 7% normal grade FCS and BrDU at a final concentration of 25µg/ml) and grown overnight at 37°C in a 5% CO_2 environment. The following day a master stock of a virus in a 3ml volume per flask was added to near confluent cells at a m.o.i. of 0.1 pfu/cell. After a one hour infection period at 37°C/ 5% CO_2, 30ml of medium was added to each flask and then incubated as before for 2-3 days. After this time the cells were collected and a virus-containing lysate prepared by three cycles of sonication and freeze/thawing, followed by a trypsin digestion. Aliquots of the resulting virus working stock were then made and stored at -80°C. The virus titre of the stock was determined by titration on 143 TK^- cells as described below.

- Arenaviruses

BHK cells were set up in flasks at ~5x10^6 cells/flask in a volume of ~30ml medium (Dulbecco's MEM supplemented with 7% normal grade FCS and 10% tryptose phosphate broth) and grown at 37°C in a 5% CO_2 environment until ~ 40% confluent (typically overnight). Cells were infected with a master stock of virus at a m.oi of 0.1 pfu/cell in a 4ml volume per flask. After a one hour infection period at 37°C/ 5% CO_2, 30ml of medium was added to each flask and then incubated as before for 2 days. After this time the virus-containing supernatant was harvested, and any cell debris removed by centrifugation. Aliquots of the resulting virus working stock were then made and stored at -80°C. The virus titre of the stock was determined by titration on Vero cells as described below.

2.2.14 (b) Plaque assay

- Recombinant vaccinia viruses

143 TK^- cells were plated out into 6 well plates at 3x10^5 cells per well in a 3ml volume and grown overnight at 37°C in a 5% CO_2 environment. The following day cells were checked to ensure they had formed a just confluent monolayer, the medium removed from the cells, and 500µl per well of one of a series of dilutions of a virus stock to be titrated (typically 1/10^3 down to 1/10^7) added to each well. One well, to which medium alone was added, was kept as a negative control. The cells were incubated with the virus for one hour at 37°C/5% CO_2 to allow infection to take place, and then overlayed with 4ml per well of a 1:1 mixture of 1% agarose in water and 2x 199 medium supplemented with 10% high grade FCS. The plates were then incubated at 37°C in a 5% CO_2 environment. After three days the cell monolayers were fixed by adding ~3ml per well of 9.25% formalin (1 in 4 dilution of a 37% stock in PBS) and leaving for one hour at room temperature. The plaques in the monolayers were visualised by discarding the agarose overlay and adding 2ml of a 1 in 10 dilution of a stock solution of 1% crystal violet to each well, and allowing the cells to uptake the dye before rinsing away excess dye. The plaques were counted and the titre of the virus stock calculated in pfu/ml as the number of plaques x dilution factor x 2.

- Arenaviruses

Vero cells were plated out into 6 well plates at $3x10^5$ cells per well in 3ml medium (Eagle's MEM supplemented with 7% normal grade FCS) and grown overnight at 37°C in a 5% CO_2 environment. The medium was removed from the cells, and 500µl per well of one of a series of dilutions of a virus stock to be titrated (typically $1/10^3$ down to $1/10^7$) added to each well. One well, to which medium alone was added, was kept as a negative control. The cells were incubated with the virus for one hour at 37°C/5% CO_2 to allow infection to take place, and then overlayed with 4ml per well of a 1:1 mixture of 1% agarose in water and 2x 199 medium supplemented with 10% high grade FCS. The plates were then incubated for six days at 37°C in a 5% CO_2 environment. The cell monolayers were then fixed for one hour at room temperature using ~3ml per well of 9.25% formalin (1 in 4 dilution of a 37% stock in PBS), after which they were stained by discarding the agarose overlay and adding 2ml of a 1 in 10 dilution of a stock solution of 1% crystal violet to each well. The cells were allowed to uptake the dye before rinsing away excess dye. The plaques were counted and the titre of the virus stock calculated in pfu/ml as the number of plaques x dilution factor x 2.

2.2.15 Preparation of anti-CD4 antibody used for murine *in vivo* depletion experiments

Immunoglobulins were enriched and concentrated from ascites fluid containing monoclonal antibody GK1.5 by ammonium sulphate precipitation. One volume of cold saturated ammonium sulphate solution was added dropwise to 2 volumes of ascites fluid on ice whilst stirring. The mixture was allowed to stand on ice for 15 mins, and then centrifuged at 2200rpm/10000g on a Sorvall RT7 Plus benchtop centrifuge (DuPont Ltd, Stevenage, UK) at 4°C. The sediment was dissolved in a small volume of PBS, injected into a dialysis cassette and dialysed against PBS with constant stirring at 4°C for ~ 24 hrs, with several changes of PBS. The antibody was harvested from the cassette, its concentration determined by protein assay as described below, and then it was stored at -20°C until use.

2.2.16 Determination of antibody concentration in ammonium sulphate precipitated ascites fluid

The concentration of antibody was determined using a Coomasie Plus 200 Protein assay in conjunction with BSA standards. The standard microplate protocol (working range 100-1500µg/ml) was followed as described below.

Protein standards were prepared by diluting a 2mg/ml BSA stock with PBS to concentrations ranging between 0 and 2mg/ml. 10µl of standard or appropriately diluted unknown sample was added to micoplate wells in duplicate. 300µl of the Coomasie Plus reagent were added to each well, and mixed for 30 secs before reading the absorbance at 595nm using a Spectramax 340 plate reader (Molecular Devices, Winnersh, UK). The average reading from the blank replicates (0µg/ml) was subtracted from the average readings of all other standards and unknowns and a standard curve prepared using SOFT max PRO version 3.1.2 software. The protein concentration in the antibody preparation was determined using the plotted standard curve.

2.2.17 Experiments in murine model systems – immunisaton protocols:

- Epitope hierarchy of the LCMV-specific $CD8^+$ response induced following LCMV infection of CD4 depleted C57BL/6 mice

On day -1 and 2 mice were inoculated by an intravenous (i.v.) route with 1mg anti-CD4 antibody in a 200µl volume. On day 0, the CD4-depleted mice plus a control group which had not received any depleting antibody were infected with $2x10^5$ pfu LCMV Armstrong by an intraperitoneal (i.p.) route in a 200µl volume. On day 8 mice were bled from a tail vein and then sacrificed, and their spleens removed for experimental analysis.

- Epitope hierarchy of the LCMV-specific $CD8^+$ response induced following VV-LCMV infection of control C57BL/6 and IA k-o mice

Normal C57BL/6 mice and IA k/o mice (also on a C57BL/6 background) were infected i.p. with a mixture of 10^6 pfu vvNP and 10^6 pfu vvGPlong in a total volume of 200µl. 8 days later the mice were sacrificed and their spleens removed for experimental analysis.

- Epitope hierarchy of the influenza-specific $CD8^+$ response induced following i.p. infection of control C57BL/6 and IA k/o mice

Normal C57BL/6 mice and IA k/o mice (also on a C57BL/6 background) were infected i.p. with 600 haemagglutinating units of influenza PR8 virus. 8 days later the mice were sacrificed and their spleens removed for experimental analysis.

- Expansion of cross-reactive memory cells in Pichinde virus-immune mice in the response to LCMV infection induced under conditions where CD4 help is limiting

Five groups of C57BL/6 mice were used in this experiment, each receiving one or more viruses ± anti-CD4 antibody. Each mouse in groups 1-3 were each infected with $2x10^5$ pfu Pichinde virus i.p in a 200µl volume. Four weeks later, on day -1, groups 3 and 5 were transiently depleted of $CD4^+$ cells by i.p. injection of 1mg anti-CD4 antibody. On day 0 groups 2-5 were challenged with $2x10^5$ pfu LCMV Armstrong given i.p. in a 200µl volume. A further inoculation of anti-CD4 antibody was given as before to mice in groups 3 and 5 on day 2. On day 8 all mice were sacrificed and their spleens removed for experimental analysis.

- Expansion of memory cells versus naïve cells in the help-dependent $CD8^+$ T cell response to the male antigen H-Y

Five groups of female C57BL/6 mice were used in this experiment. Mice in groups 1-3 were each given an inoculation of $5x10^6$ cells from a splenocyte suspension prepared from male C57BL/6 mice. Mice in groups 4 and 5 were not initially primed. Four weeks later on day -1, mice in groups 2 and 5 were given an inoculation of 1mg anti-CD4 antibody i.v. in a 200µl volume. On day 0, mice in all groups except for those in group 3 were given a primary or boosting inoculation of $5x10^6$ cells from a splenocyte suspension prepared from male C57BL/6 mice. Mice in groups 2 and 5 were kept CD4 cell-deficient by further inoculations of anti-CD4 antibody as before on days 2 and 7. All mice were sacrificed, and their spleens removed for experimental analysis on day 13.

2.2.18 Murine IFN-γ ELISPOT assay

Multiscreen 96-well filtration plates were coated overnight at 4°C with 100µl per well of capture antibody (purified anti-IFN-γ mAb clone R4-6A2) diluted to

5µg/ml in 0.1M sodium bicarbonate buffer, pH9.6). Plates were washed three times with filtered PBS and blocked by the addition of 200µl per well RPMI 1640 supplemented with 10% normal grade FCS for at least 2 hrs at 37°C in a 5% CO_2 environment. Plates were washed again before the addition of cells.

Single cell suspensions were prepared from the spleens of experimental mice by a process involving mechanical disruption of splenic tissue, removal of red cells using red cell lysis buffer and removal of clumps by passing the suspensions through cells sieves. Cells were washed three times in RPMI 1640 supplemented with 2% FCS, counted, resuspended to 10^7c/ml, and diluted so between $2x10^5$ and $2x10^3$ cells could be added per well in a 100µl volume. An aliquot of each suspension (~10^6 cells) was stained with a fluorochrome-conjugated anti-CD8 antibody, and the percentage of $CD8^+$ T cells determined by flow cytometry so that ELISPOT results could be expressed as SFC per 10^6 $CD8^+$ T cells. Feeder cells were prepared from the spleens of control mice in a similar way, irradiated at 3000 Rads using a Gammacell 1000 Elite irradiator (Nordion International Inc.), washed and resuspended to a concentration of $2x10^6$c/ml so that 10^5 feeder cells could be added per well in a 50µl volume. Recombinant human IL-2 was also added at 10IU/well. Peptides were added in a 50µl volume to give a final concentration of 10^{-6}M. Positive and negative control wells were included in all assays; in these wells con A (final concentration 2.5µg/ml) or medium only were added in place of peptide. All variables were assayed in duplicate. Plates were incubated overnight at 37°C in a 5% CO_2 environment.

To detect bound IFN- γ, plates were washed three times with filtered PBS and three times with PBS containing 0.05% Tween-20 before the addition of 100µl secondary antibody (biotinylated anti-IFNγ mAb clone XMG1.2 diluted to a final concentration of 1µg/ml). They were then incubated overnight at 4°C. Plates were washed five times with PBS-Tween, then 100µl per well of anti-biotin AP was added (final concentration 1µg/ml) and the plates were incubated for 4hrs at 37°C. Plates were washed four times with PBS-Tween and four times with PBS. After the final wash, plates were taken apart to wash the back of the filters to remove trapped antibody. Alkaline phosphatase substrate was prepared by diluting a colour development buffer to a 1x solution in sterile water and adding 100µl AP colour reagents A and B per 10ml. 100µl substrate was added per well and plates were allowed to develop

for 30 mins at room temperature. The reaction was stopped by discarding the substrate and rinsing the filters under tap water. Plates were allowed to dry overnight and spots counted using an AID automated image analysis system with AID ELISPOT version 2.3 software.

Results were calculated from spot values obtained at the most appropriate cell density and are expressed as the mean number of SFC per 10^6 $CD8^+$ cells. This was calculated by finding the mean of replicate wells and deducting the mean number of spots counted in the negative control wells, then scaling up based on the percentage of $CD8^+$ cells in the sample and number of cells per well.

2.2.19 Staining murine splenocytes with antibodies to cell surface markers

In 96-well plates, ~10^5 cells per sample were pelleted and relevant mAbs to cell surface markers added in a 50µl volume and incubated for 15 mins at 4°C. Cells were then washed three times and fixed using 200µl 4% paraformaldehyde. Samples were run on a FACS Calibur (Beckton Dickinson, Oxford, UK) and data was analysed using Cell Quest Pro software.

2.2.20 H-Y tetramer staining of murine splenocytes

Splenocyte suspensions were prepared as described above, washed and the cells pelleted. To remove B cells, 100µl/spleen of sheep anti-mouse IgG Ab Dynalbeads were added, mixed and left at room temperature for 30 mins. 5ml of PBS supplemented with 2% FCS was then added to each sample, and labelled B cells were removed by magnetic separation. The negative fraction was collected, washed and counted. 10^6 cells in 50µl were stained with 1µl D^b-Uty-PE tetramer for 20 mins at room temperature. Cells were then stained with CD8-Cy5 antibody for 20 mins on ice, washed twice and resuspended in PBC/2% FCS without fixing. Samples were run on a FACS Calibur and data was analysed using Cell Quest Pro software.

2.2.21 Combined LCMV tetramer and TCR Vβ region staining of murine blood lymphocytes

Blood samples were collected from mice by making an incision in a tail vein, and collecting 6-8 drops of blood into a tube containing 200µl blood buffer (10mM EDTA and 100IU heparin/ml in PBS). Red cells were removed by the addition of 1ml red cell lysis buffer per sample and incubation for 15 mins at

room temperature, followed by centrifugation, to leave a white pellet of lymphocytes. To reduce FcR-mediated binding of antibodies, 2µl Fc block was added to the pellet for 10 mins on ice. Cells were then resuspended in an appropriate volume of FACS buffer (PBS supplemented with 2% BSA), and transferred to wells of a 96 well round bottomed plate, with cells in a volume of 50µl per well. Cells were then co-stained with optimised quantities of fluorescently labelled tetramer, anti-CD8 antibody and one of a panel of Vβ-specific antibodies. (Each tetramer had been tested on cells using different concentrations, temperatures and incubation times to determine the minimum concentration and combination of conditions which gave the brightest specific staining and lowest background staining.) Staining was carried out for 45 mins at room temperature, after which cells were washed twice in FACS buffer and fixed in 4% paraformaldehyde. Samples were run on a FACS Calibur and data was analysed using Cell Quest Pro software.

Chapter 3

Characterisation of the breadth and specificity of the virus-specific $CD8^+$ response in primary HIV-1 infection

3.1 Introduction

As reviewed in chapter 1, it is known that the virus-specific $CD8^+$ T cell response plays an important role in control of viral replication during early HIV-1 infection although, at least in the vast majority of infected individuals, it fails to contain viral replication completely. A better understanding of the nature of the $CD8^+$ response mounted to HIV-1 during the early stages of infection may help to explain why this response is not more effective in controlling viral replication. However, there have been relatively few studies of the HIV-specific $CD8^+$ T cell response during primary infection (Appay, 2002; Appay *et al.*, 2002; Borrow *et al.*, 1994; Borrow *et al.*, 1997; Cao *et al.*, 2003; Dalod *et al.*, 1999b; Doisne *et al.*, 2004; Goulder *et al.*, 2001a; Koup *et al.*, 1994; Musey *et al.*, 1997; Papagno *et al.*, 2004; Price *et al.*, 1997; Safrit *et al.*, 1994; Wilson *et al.*, 2000). The overall magnitude, breadth and kinetics of expansion of the response thus remain poorly characterised, and whether/how these parameters may vary in different infected individuals is not well understood.

The aim of this study was to explore such features of the primary HIV-specific $CD8^+$ T cell response in a panel of HIV seroconverters who naturally (in the absence of early antiretroviral therapy) established differing persisting viral loads (see section 3.2), and to determine whether there was any association between the magnitude, breadth and/or specificity of the response and the persisting viral load established.

Traditionally, $CD8^+$ T cell responses were detected using lytic assays, which measure the ability of populations of cells (either *ex vivo* cells or those expanded during a period of *in vitro* culture) to lyse radiolabelled, antigen-pulsed, HLA-matched target cells. Limiting dilution assays were employed to obtain more quantitative results: here CTL precursor frequencies were calculated after measuring the antigen-specific lytic potential of cells expanded in limiting dilution cultures. However, these assays have a number of drawbacks, including not being very quantitative or sensitive, problems associated with *in vitro* culture of cells and being relatively time-consuming to perform. Newer improved assays now allow for easier, more sensitive and accurate assessment of antigen-specific $CD8^+$ responses (Doherty, 1998; McMichael & O'Callaghan, 1998). These include assays which detect

responding T cells on the basis of cytokine production following antigenic stimulation (such as ELISPOT assays or intracellular cytokine staining (ICS) assays) and those which involve staining cells with fluorescently tagged multimeric complexes of an antigenic peptide and MHC allele. ELISPOT assays and ICS can both be used to screen rapidly for responses to different proteins/peptides and have the added advantage that no prior information about the precise epitopes or their MHC restriction is needed. However, because they employ a functional readout to detect responding cells, they do not reveal the exact magnitude of responses made as they cannot detect cells that are unable to mount the response being measured (e.g. produce a particular cytokine) following antigenic stimulation. The use of tetramers to detect antigen-specific $CD8^+$ T cells allows for a more precise measurement of the magnitude of responses, but has the drawback that the epitope and its MHC restriction have to be known, plus the cost of purchasing tetramers limits the number of epitopes that can be feasibly studied.

Here it was initially chosen to use ELISPOT assays to assess patients' $CD8^+$ T cell responses, first studying responses to whole HIV proteins which were presented by a viral vector, and then assessing responses to overlapping synthetic 20-mer peptides corresponding to the entire HIV-1 clade B consensus sequence. These assays allowed rapid screening of responses to different HIV proteins, such that information about the specificity and breath of the HIV-specific $CD8^+$ T cell response in multiple patients could be obtained. As described in chapter 5, I subsequently went on to characterise individual epitope-specific responses identified using the ELISPOT assays more comprehensively, using tetramer staining. This enabled the magnitude of specific responses to be followed over a series of timepoints, and when used in combination with staining with other fluorescently labelled antibodies, the breadth of TCR Vβ family usage in individual epitope-specific responses and the phenotype of the T cells could be explored.

In summary, in this chapter I used ELISPOT assays to analyse the epitope breadth and specificity of the HIV-specific $CD8^+$ T cell response raised during primary infection in a number of patients. Also included at the end of the chapter is some analysis of the TCR Vβ family usage by epitope-specific T cells, which I carried out to try and gain some insight into the clonal breadth, as well as the epitope breadth, of the HIV-specific $CD8^+$ T cell response in primary infection. The main objectives of this work were to learn more about the nature of the primary HIV-specific $CD8^+$ T cell response and to determine

whether/how the nature of the response may differ in patients who control viral replication with differing efficiency, establishing different persisting viral loads.

3.2 HIV seroconverter cohort

The PBMC samples used in this study were obtained from a cohort of HIV seroconverters recruited by Dr Ian Williams from the Mortimer Market Centre (London, UK), with the exception of samples from patient SC1 (obtained from Dr George Panayiotakopoulos, St George's Hospital Medical School, London) (patients from the latter two sources are collectively referred to as the London cohort of patients) and SUMA (obtained from Dr. George Shaw, University of Alabama at Birmingham, USA). The clinicians concerned provided details of each patient's clinical history and disease course, together with sequential viral load and CD4 count measurements. Clinical tests suggested that all subjects were infected with the clade B subtype of HIV-1. All patients presented with symptomatic primary HIV infection, with symptoms such as nausea, vomiting, headaches, fever, sore throat, rash and lymphadenopathy. Peripheral blood samples were collected from patients at sequential timepoints after diagnosis, and were assigned a timepoint in accordance with the number of days following onset of symptoms (DFOSx) that the sample was taken. Although others may define the various phases of HIV infection differently, throughout this thesis the following definitions are used: acute infection - the time at which the burst of acute viral replication occurs, before detection of HIV-1 antibodies by ELISA or Western immunoblot (typically 0-2 weeks FOSx); subacute infection - when the acute viral burst starts to be contained, and during which the patient is antibody indeterminant (typically 3-4 weeks FOSx); early infection – after full serocoversion (typically between 5 weeks FOSx and approximately 6 months FOSx); and chronic infection - any time thereafter. Primary infection is used to define the period encompassing the acute, subacute and early phases of infection. Because the day of symptom onset is based on patient histories and may not be completely accurate in all cases, the viral load may provide a better indicator of the time within primary infection at which sample collection was begun. For some patients, samples were obtained coincident with the peak of viral replication, but for many patients, sample collection began after the acute burst of viral replication, more coincident with the early phase of infection onwards.

A list of the patients studied and some relevant clinical data is shown in Table 3.1. Their HLA types are given in Table 3.2. The patients have been divided

Table 3.1 Clinical profiles of the patients used for T cell studies in chapter 3.

Viral load quartile[1]	Patient	Persisting viral load established (RNA copies/ml)[2]	Subsequent disease course
High	MM24	170,200 at 198 DFOSx	Has maintained a stable CD4 cell count for the duration of follow-up (>2.5 years)
	MM23	117,600 at 204 DFOSx	Maintained a stable CD4 cell count until lost to follow up (~ 18 months FOSx)
	MM25	71,100 at 185 DFOSx	Maintained a stable CD4 cell count until lost to follow up (~ 22 months FOSx)
	SC1	67,075 at 7 months FOSx	Has maintained a stable CD4 cell count for the duration of follow-up (>1.5 years)
	MM26	44,600 at 169 DFOSx	Maintained a stable CD4 cell count until lost to follow up (~ 17 months FOSx)
Intermediate-high	MM12	34,900 at 230 DFOSx	CD4 cell count fell to 200 x 10^6 cells/l by 899 DFOSx; patient subsequently received HAART at 908 DFOSx. Viral load has been maintained at <50 RNA copies/ml since initiation of therapy.
	MM9	30,900 at 189 DFOSx	CD4 cell count fell to <200 x 10^6 cells/l by 876 DFOSx; patient subsequently received HAART. Viral load has been maintained at <50 RNA copies/ml and CD4 cell counts >200 x 10^6 cells/l since initiation of therapy.
	MM4	30,200 at 206 DFOSx	CD4 cell count fell to 390 x 10^6 cells/l by the time which the patient was subsequently lost to follow-up (~4 years)
	MM27	28,800 at 202 DFOSx	Has maintained a stable CD4 cell count for the duration of follow-up (~2 years)
	MM14	27,200 at 179 DFOSx	Has maintained a stable CD4 cell count for the duration of follow-up (> 3 years)

	MM19	13,900 at 192 DFOSx	Diagnosed with acute Hepatitis C virus infection at 95 DFOSx. Subsequently received IFN and ribavirin treatment. Hepatitis C virus undetectable by 287 DFOSx. Stable CD4 counts maintained throughout.
Low-intermediate	MM35	9,600 at 192 DFOSx	Has maintained a stable CD4 cell count for the duration of follow-up (>1 year)
	MM13	8,200 at 196 DFOSx	Has maintained a stable CD4 cell count for the duration of follow-up (>3 years)
	MM28	5,600 at 198 DFOSx	Has maintained a stable CD4 cell count for the duration of follow-up (>2 years)
Low	SUMA	2,268 at 278 DFOSx	Maintained a stable CD4 cell count for >7 years; patient subsequently lost to follow-up

N.D. [3]	MM22	N.D. [3] Last recorded viral load before commencement of therapy – 3,249,700 at 25 DFOSx	Received HAART for a 6 month period from 26-196 DFOSx. Has maintained a stable CD4 cell count since coming off therapy for the subsequent duration of follow-up (>1.5 years).
	MM18	N.D. [3] Last recorded viral load – 680,500 at 57 DFOSx	Patient lost to follow-up after ~ 8 months FOSx.
	MM11	N.D. [3] Last recorded viral load before commencement of therapy – 355,700 at 25 DFOSx	Received HAART from 29-426 DFOSx; then resumed therapy again after 990 DFOSx.

N.A [4]	DO	N.A [4] 3,200 at 27 months post-diagnosis	This patient consented to giving samples at three timepoints during chronic infection (~ 2-4 years after diagnosis) and was not monitored over the course of primary infection.

[1] Patients are listed according to the "setpoint" persisting viral load, i.e. the persisting viral load they established by ~6 months FOSx (the viral load measurement of the sample taken nearest to 180 DFOSx). This column indicates which of the four viral load quartiles identified in a study by Mellors *et al* (Mellors *et al.*, 1996) the patients fall into: low (<4531 RNA copies/ml plasma), low-intermediate (4,531-13,020 RNA copies/ml plasma), intermediate-high (13,020-36,270 RNA copies/ml plasma), or high (> 36,270 RNA copies/ml plasma) persisting viral load.

[2] Plasma viral RNA load at ~6 months FOSx (the viral load measurement of the sample taken nearest to 180 DFOSx). The timepoint at which the viral load given was recorded is also indicated.

[3] N.D. Not determined, as these patient went onto antiretroviral therapy before 6 months.

[4] N.A. Not applicable. This patient was not followed over the period of seroconversion through to the time of establishment of the persisting viral load, and so has not been categorised as the other patients have been.

Table 3.2. HLA types of patients used for T cell studies in chapter 3.

Viral load quartile[1]	Patient	HLA type[2]		
High	MM22	A*02	B*15	Cw*03
		A*24	B*51	Cw*16
	MM18	A*02	B*08	Cw*05
		A*02	B*44	Cw*07
	MM11	N.D.	N.D.	N.D.
	MM24	A*01	B*08	Cw*01
		A*02	B*51	Cw*07
	MM23	A*02	B*44	Cw*05
		A*23	B*49	Cw*07
	MM25	A*11	B*07	Cw*07
		A*03	N.D.	N.D.
	SC1	A*01	B*08	Cw*05
		A*02	B*44	Cw*07
	MM26	A*02	B*51	Cw*15
		A*68	B*35	Cw*04
Intermediate-high	MM12	A*03	B*07	Cw*07
		A*68	B*44	Cw*07
	MM9	A*01	B*41	Cw*07
		A*66	B*08	Cw*17
	MM4	A*02	B*44	Cw*07
		A*29	B*58	Cw*16
	MM27	A*02	B*07	Cw*05
		A*03	B*44	Cw*07
	MM14	A*02	B*27	Cw*02
		A*01	B*08	Cw*07
	MM19	A*01	B*44	Cw*06
		A*68	B*57	Cw*07
Low-intermediate	MM35	A*01	B*35	Cw*12
		A*24	B*39	Cw*11
	MM13	A*01	B*08	Cw*06
		A*01	B*57	Cw*07
	MM28	A*11	B*13	Cw*04
		A*30	B*35	Cw*06
Low	SUMA	A*11	B*14	Cw*08
		A*24	B*15	Cw*12

N.D. Not determined

[1] Patients are grouped according to the viral load they established at ~6 months FOSx (see Table 3.1)

[2] The MHC class I types of all patients studied was determined by the Oxford Transplant Centre, UK, using a PCR based technique.

into four groups according to the viral load that they established at around six months FOSx, with patients being defined as having either a low (<4531 RNA copies/ml plasma), low-intermediate (4,531-13,020 RNA copies/ml plasma), intermediate-high (13,020-36,270 RNA copies/ml plasma), or high (> 36,270 RNA copies/ml plasma) setpoint viral load. These viral load ranges are taken from a study by Mellors *et al* in which the relationship between the persisting viral load established by ~six months post-infection and the subsequent disease course was addressed in a cohort of 180 HIV-infected individuals in the USA (Mellors *et al.*, 1996). In this cohort, these viral load ranges divided the patients into four quartiles. The time to development of AIDS and death of patients in these viral load quartiles was shown to differ, with each group having a different median survival time (see Figure 1.5). The patients within the cohort I studied were not evenly distributed between the four groups, with the majority establishing persisting viral loads which were high or intermediate-high: this is discussed further at the end of the chapter.

3.3 Experiments to investigate the suitability of a recombinant vaccinia virus-stimulated IFN-γ ELISPOT assay for analysis of HIV-specific CD8⁺ T cell responses

To give an overview of the breadth of the functional HIV-specific $CD8^+$ T cell response at the protein level, and the relative immunodominance of responses to different proteins I initially used rVV-stimulated IFN-γ ELISPOT assays (based on a method by Larsson *et al* (Larsson et al., 1999)) to assess T cell responses to five different HIV proteins (gp160, Gag, Pol, Tat and Nef - all derived from laboratory isolates of HIV-1). In this assay PBMCs are infected with recombinant vaccinia viruses containing HIV-1 genes. During a 48-hour incubation period, cells within the PBMC population (predominantly monocytes) are infected by the vaccinia viruses and the HIV proteins they encode are expressed within these cells. HIV proteins are then processed and presented for recognition with MHC class I molecules at the cell surface and HIV-specific $CD8^+$ T cells within the PBMC population which recognise these peptide-MHC complexes are stimulated to produce IFN-γ, which is detected in a standard ELISPOT assay.

Figure 3.1 shows results from a representative rVV-stimulated IFN-γ ELISPOT assay. Both the data initially obtained and its final representation (with the background - the level of IFN-γ production seen when cells were stimulated with a control vaccinia virus - deducted) are shown to explain how the final

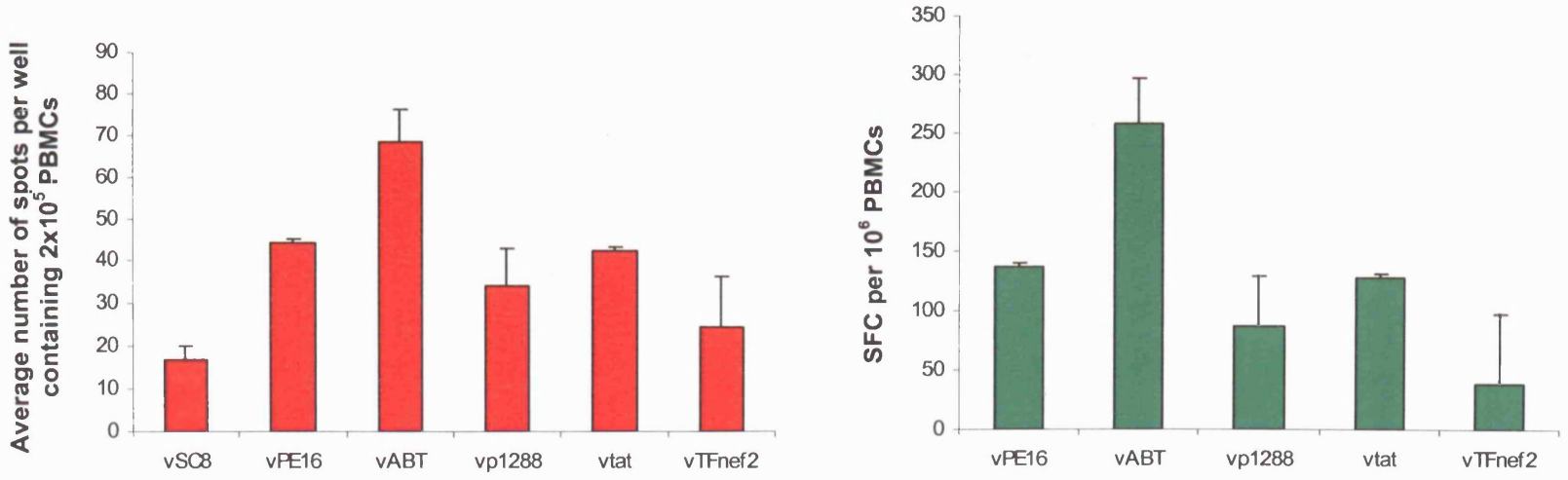


Figure 3.1. Determination of the responses made by patient MM23 to different HIV-1 proteins using a recombinant vaccinia virus-stimulated ELISPOT assay. PBMCs cryopreserved from patient MM23 at 9 DFOSx were thawed and tested for responses to recombinant vaccinia viruses expressing HIV proteins (or a control protein): vSC8 - β-gal only, vPE16 - HIV gp160, vAbT – HIV Gag, vP1288 – HIV Pol, vTat – HIV Tat and vTFNef$_2$ – HIV Nef. Cells were infected with recombinant vaccinia viruses at a moi of 2 pfu/cell and 48hrs later, the frequency of IFN-γ spot forming cells (SFC) was measured by ELISPOT assay. The graph on the left shows the average (of duplicate or triplicate wells) number of spots per well following stimulation of $2x10^5$ PBMC with the different recombinant vaccinia viruses. The graph on the right shows the results after the background value (average number of spots per well when cells were stimulated with a control vaccinia virus expressing β-gal only) was deducted from the data on the left, and values scaled up to express the data as the number of specific SFC per 10^6 PBMC. The error bars indicate 1 SD above the mean. All subsequent data from rVV-stimulated ELISPOT assays are expressed in this way.

results were calculated from the raw data. In subsequent figures, all results are shown with the background values deducted. The background values that have been subtracted are given in the figure legends to enable comparison of the magnitude of each response in relation to the background level of response. Although the background values were variable between patients and even within the same patient when cells from different timepoints were used, backgrounds were generally low (in the range of 10-625 SFC per 10^6 PBMC). If the background was very high using cells from a particular patient or timepoint (greater than 700 SFC per 10^6 PBMC), then the data was considered to be unreliable and was excluded from the analysis. High backgrounds may have been due to non-specific secretion of IFN-γ or pre-existing immunity to the vaccinia vector.

Prior to use of rVV-stimulated ELISPOT assays for assessment of HIV-specific $CD8^+$ T cell responses, experiments were carried out to confirm that the HIV-specific IFN-γ responses detected using this assay were mediated by $CD8^+$ T cells, and also to investigate the reproducibility of results obtained using this assay method.

Larsson *et al* showed that the responses they detected in HIV-infected individuals using the rVV-stimulated ELISPOT assay were indeed mediated by $CD8^+$ T cells (Larsson *et al.*, 1999). Further, previous results from our group have shown that it is difficult to detect HIV-specific $CD4^+$ T cell responses in typical HIV-infected individuals using cryopreserved cells in ELISPOT assays (Gloster *et al.*, 2004), again suggesting that the majority of responses detected in rVV-stimulated ELISPOT assays would be mediated by the $CD8^+$ T cell subset. To confirm this, CD8-depleted PBMC were tested in parallel with total PBMC for responses to the five rVVs to verify the phenotype of the responding population of cells. CD4-depleted PBMC were also tested as a control to show that if responses were abrogated by removal of $CD8^+$ cells, this wasn't just due to alteration of the cells that had been subjected to magnetic separation. In Figure 3.2 it can be seen that patient MM4 predominantly made responses to HIV gp160, Pol and Tat, plus low-level responses to Gag and Nef. Depletion of $CD8^+$ cells reduced the response detected to all proteins, with any residual response in most cases being below twice the background level. By contrast, $CD4^+$ cell depletion had little or no effect on the response observed to most proteins, the one exception being the response to gp160. Whilst this could mean that a proportion of this response was mediated by $CD4^+$ T cells, the fact that CD8 depletion also very strongly reduced this response suggests that

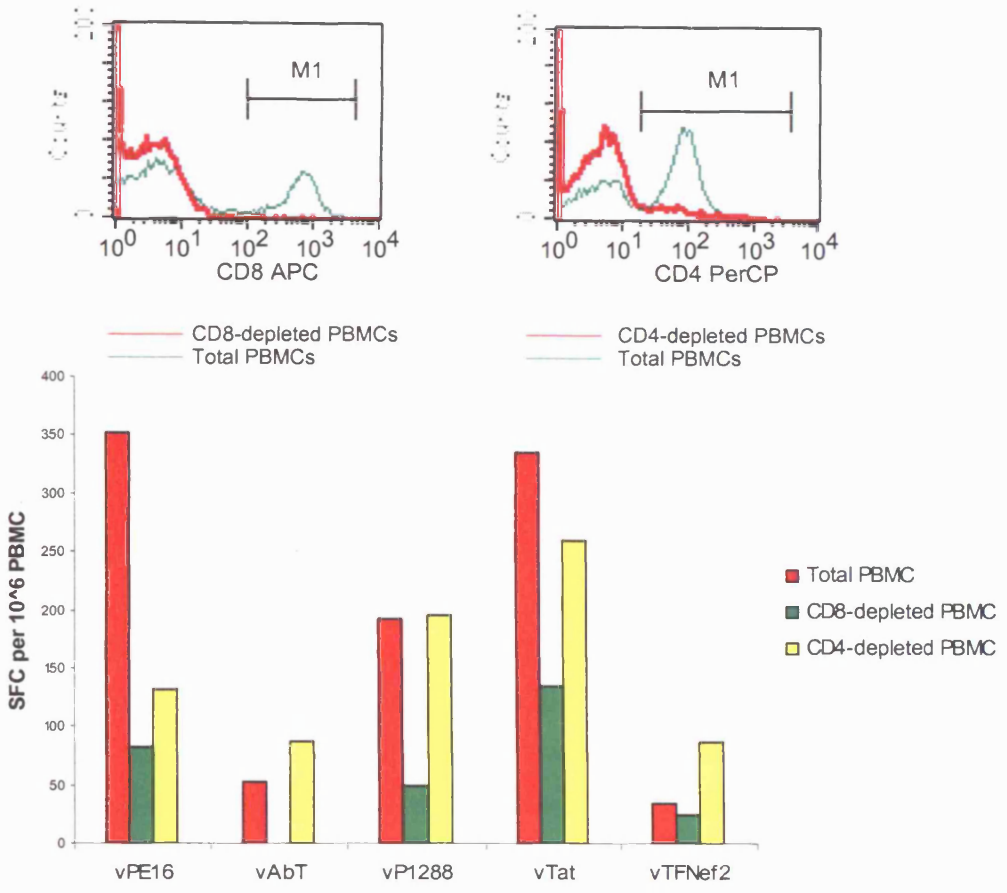


Figure 3.2. Investigation of the phenotype of the T cells mediating the HIV-specific IFN-γ response in rVV-stimulated ELISPOT assays. Cryopreserved PBMC from patient MM4 (pooled cells from 1058 and 1222 DFOSx) were thawed and either $CD8^+$ or $CD4^+$ cells removed using magnetic beads. The efficiency of the depletions was assessed by staining total, CD8-depleted and CD4-depleted cells with anti-CD8 APC and anti CD4 PerCP monoclonal antibodies. Flow cytometric analysis of the stained cells revealed that the $CD8^+$ cells in the CD8-depleted cell sample had been reduced by 99% and the $CD4^+$ cells in the CD4-depleted cell sample by 83% (top two panels). Total, CD8-depleted and CD4-depleted cells were then tested in parallel for responses to recombinant vaccinia viruses expressing HIV proteins (or a control protein): vPE16 - HIV gp160, vAbT – HIV Gag, vP1288 – HIV Pol, vTat – HIV Tat and $vTFNef_2$ – HIV Nef and vSC8 - β-gal only. Cells were infected with recombinant vaccinia viruses at a moi of 2 pfu/cell and 48hrs later, the frequency of IFN-γ spot forming cells (SFC) was measured by ELISPOT assay. Results are expressed as the mean (of duplicate wells) number of SFC per 10^6 PBMC after deduction of the background value (mean number of SFC generated when cells were infected with the control rVV). The background values were 60, 60 and 15 SFC/10^6 PBMC for the total, CD8-depleted and CD4-depleted cells respectively. The results shown are representative of findings made in two independent experiments.

this was not a CD4-mediated response. Altogether, the results from this experiment suggested that the T cells producing IFN-γ in this assay were predominantly $CD8^+$ T cells.

Further experiments were carried out to investigate the reproducibility of results obtained using the rVV-stimulated ELISPOT assay. In Figure 3.3 the results from two assays carried out on separate days using two separate vials of cells cryopreserved from the same patient at the same timepoint (both cryopreserved at the same time) and aliquots of the same virus stocks are shown. The magnitudes of the responses to all of the viruses were markedly higher in assay two. Variability in the magnitude of the response observed in repeated rVV-stimulated ELISPOT assays was also seen in experiments carried out with cells from other patients too, although this is an extreme example. This variability may reflect differences in the viability of vials of cryopreserved patient cells (although these were not apparent on thawing), and/or differential survival of cells during the two day culture period in different assays. Notably, despite the inter-assay variability in the responses observed in rVV-stimulated ELISPOT assays, replicate wells in a single experiment were much less prone to variability. However, although there were inconsistencies in the magnitude of the protein-specific responses recorded in repeat assays, the hierarchy of responses to different proteins always remained the same. For example, in the two assays shown in Figure 3.3, the most dominant response was to gp160 and the weakest responses were to Gag and Nef. In the light of these observations, it was concluded that rVV-stimulated ELISPOT assays could not reliably be used for comparison of the magnitudes of HIV protein-specific responses at different timepoints and/or in different patients. However these assays could be used to address the hierarchy of responses to different proteins in different patients and at different timepoints within a given patient. To try to reduce the impact of inter-assay variability on responses observed in each patient it was decided to test cells cryopreserved from a given patient at a series of timepoints together in a single assay.

3.4 Longitudinal analysis of the HIV-specific $CD8^+$ T cell response by rVV-stimulated IFN-γ ELISPOT assay in patients who controlled early viral replication with differing efficiency

rVV-stimulated IFN-γ ELISPOT assays were used to give an overview of the relative magnitude of the T cell response to different HIV-1 proteins during

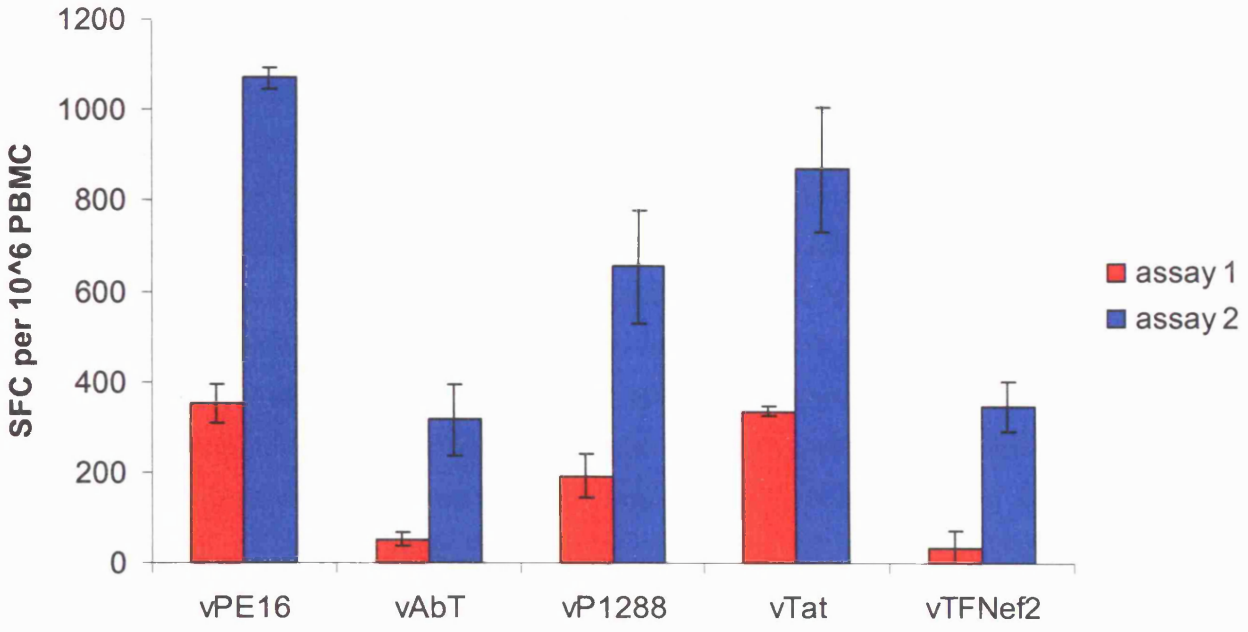


Figure 3.3. Extent of assay-to-assay variability between rVV-stimulated IFN-γ ELISPOT assays. Cryopreserved PBMCs from patient MM4 (pooled cells from 1058 and 1222 DFOSx) were stimulated for 48 hours with recombinant vaccinia viruses encoding HIV-1 proteins (vPE16 - HIV gp160, vAbT – HIV Gag, vP1288 – HIV Pol, vTat – HIV Tat and $vTFNef_2$ – HIV Nef) at a m.o.i. of 2 pfu/cell. The number of cells stimulated to produce IFN-γ was then enumerated by ELISPOT assay and is expressed as the mean (of duplicate or triplicate wells) number of SFC per 10^6 PBMC after deduction of background values (the number of cells found to produce IFN-γ following stimulation with a recombinant vaccinia virus encoding β-gal only). The background values were 60 and 290 SFC per 10^6 PBMC for assays 1 and 2 respectively. The red and blue bars represent results of two independent experiments using cells cryopreserved from the same patient/timepoint, and the viruses diluted from the same stocks. The error bars indicate 1 SD above and below the mean.

primary infection in different HIV-infected individuals. The number of cells producing IFN-γ in response to stimulation by vaccinia viruses encoding HIV gp160, Gag, Pol, Nef and Tat was determined at sequential timepoints over the course of acute, subacute (where samples were available), and early infection and at later timepoints in two patients who established high persisting viral loads (Figure 3.4), four patients who established intermediate-high viral persisting loads (Figure 3.5), three patients who established low-intermediate persisting viral loads (Figure 3.6) and one patient who established a low persisting viral load (Figure 3.7). Some patients went onto antiretroviral therapy by early infection, but all data shown is from experiments using cells cryopreserved at timepoints when patients were not on therapy. Viral load data are shown for each patient and, as mentioned before, are the best indicator of the stage of infection. For a number of the patients here, samples from timepoints coincident with the acute viral burst were not available, and the samples are from timepoints corresponding to subacute/early infection onwards. All graphs show the number of IFN-γ spot forming cells per 10^6 PBMC after deduction of background values (IFN-γ production by cells stimulated with a recombinant vaccinia virus expressing β-gal only). The y-axis scales used on different graphs are different because, as discussed in section 3.3, due to potential variation in the magnitude of the response seen in different assays, reliable conclusions cannot be drawn from this data about the absolute magnitude of responses and how this may differ in one patient compared to another.

In every patient, HIV-specific T cell responses were detected from the earliest timepoint available for testing, which in several patients was at/near the peak in acute viral replication and in patient SUMA was as early as 4 DFOSx. This demonstrates that HIV-specific $CD8^+$ T cell responses start to be induced very rapidly after infection. The kinetics of expansion of protein-specific responses could not be reliably determined using this assay, but it was notable that in several patients (e.g. MM22, MM19, MM9 and MM27) the responses detected at the earliest timepoint(s) tested appeared much weaker than those observed subsequently. This could potentially reflect poor *in vitro* survival of cells highly activated *in vivo* in the presence of a high antigenic load; and/or relatively delayed expansion of HIV-specific $CD8^+$ T cell responses in these individuals (who, notably, all established high or intermediate-high persisting viral loads). This issue is addressed in more depth in chapter 5.

In the majority of patients tested, the response observed was heavily biased

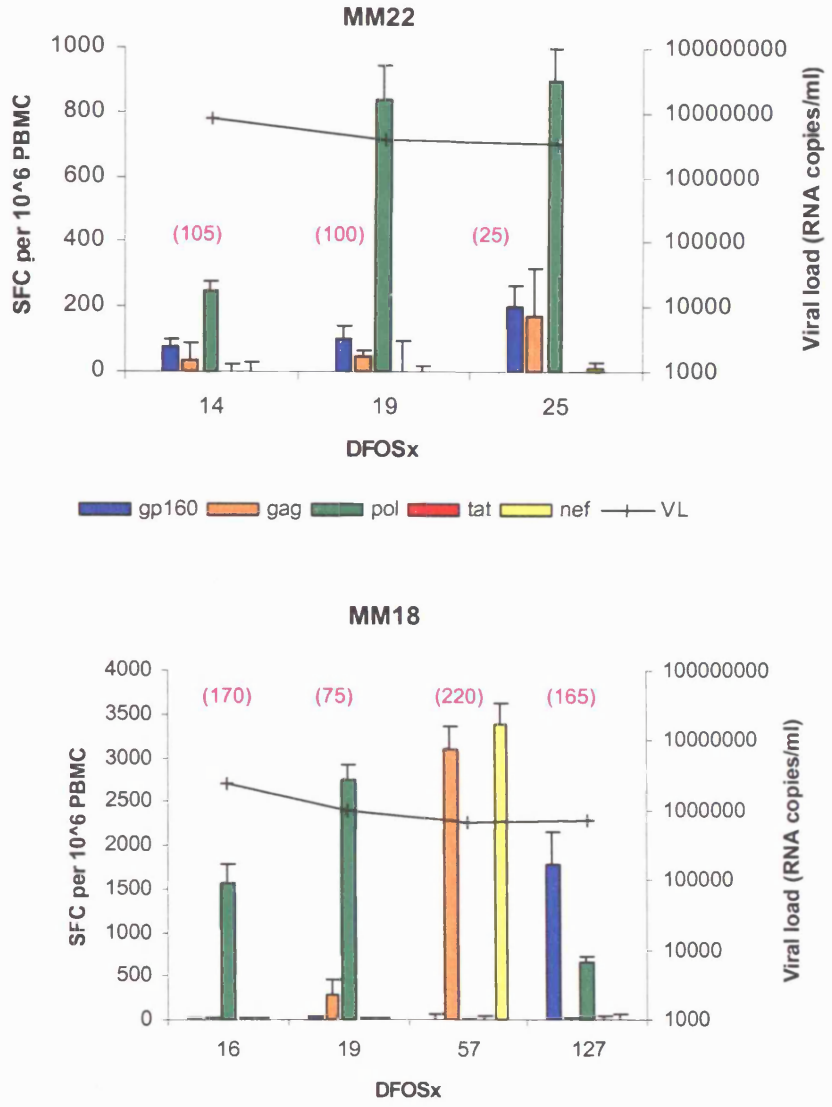
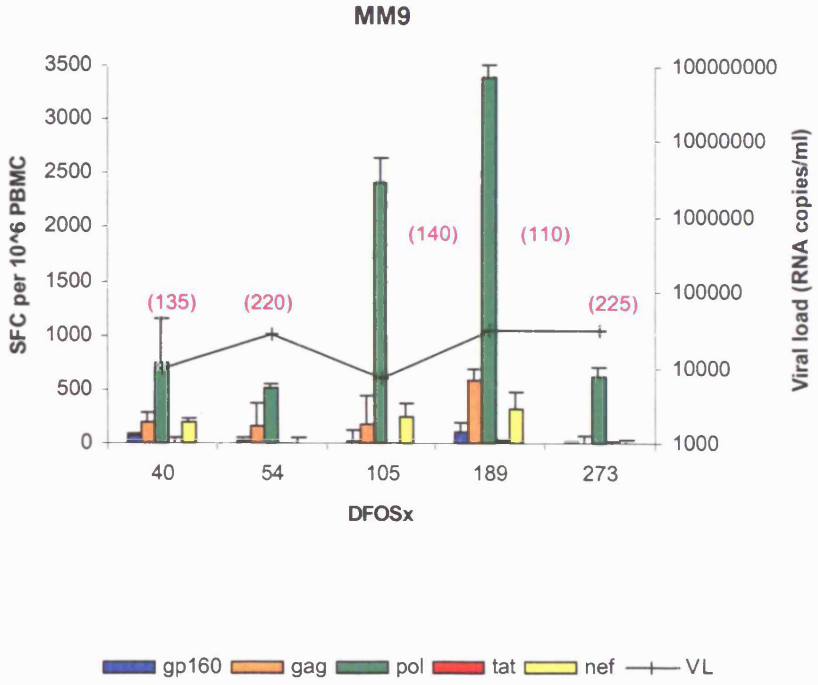
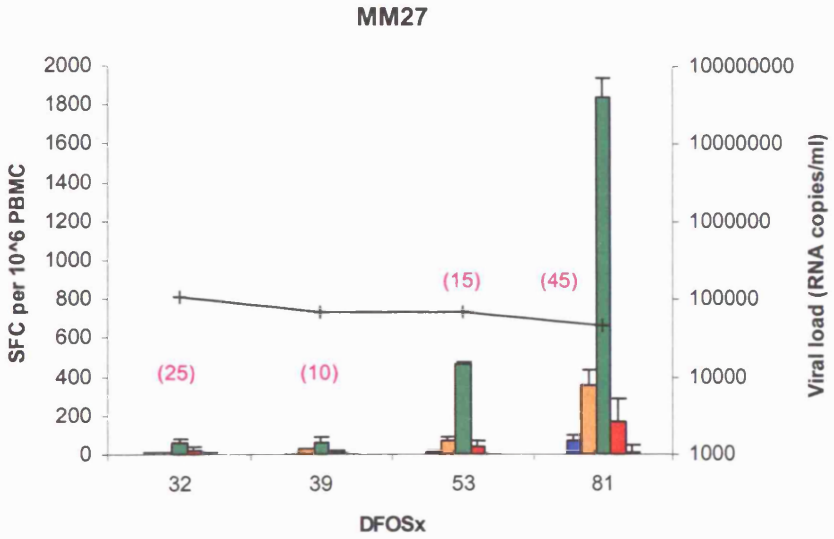


Figure 3.4. Analysis of HIV-specific T cell responses by recombinant vaccinia-stimulated IFN-γ ELISPOT assay during primary infection in HIV-infected individuals who established high setpoint viral loads. PBMCs cryopreserved from patients MM22 and MM18 at sequential timepoints during infection were stimulated in duplicate or triplicate wells for 48 hours with recombinant vaccinia viruses expressing HIV proteins (gp160, Gag, Pol, Tat and Nef) or a control protein (β-gal), and the number of cells that produced IFN-γ was assessed by ELISPOT assay. Frequencies are expressed as the mean (of duplicate or triplicate wells) number of spot forming cells per 10^6 PBMC after deduction of background values (the number of cells found to produce IFN-γ following stimulation with a recombinant vaccinia virus encoding β-gal only). Figures in brackets indicate the background values (expressed as the number of spot forming cells per 10^6 PBMC) at each timepoint. The error bars indicate 1 SD above the mean. The viral load values shown (HIV RNA copies/ml) were provided by Dr. I. Williams (Mortimer Market Centre, UK).


MM9
SFC per 10^6 PBMC
Viral load (RNA copies/ml)
DFOSx
3500
3000
2500
2000
1500
1000
500
0
100000000
10000000
1000000
100000
10000
1000
40
54
105
189
273
(135)
(220)
(140)
(110)
(225)
gp160
gag
pol
tat
nef
VL


MM27
SFC per 10^6 PBMC
Viral load (RNA copies/ml)
DFOSx
2000
1800
1600
1400
1200
1000
800
600
400
200
0
100000000
10000000
1000000
100000
10000
1000
32
39
53
81
(25)
(10)
(15)
(45)

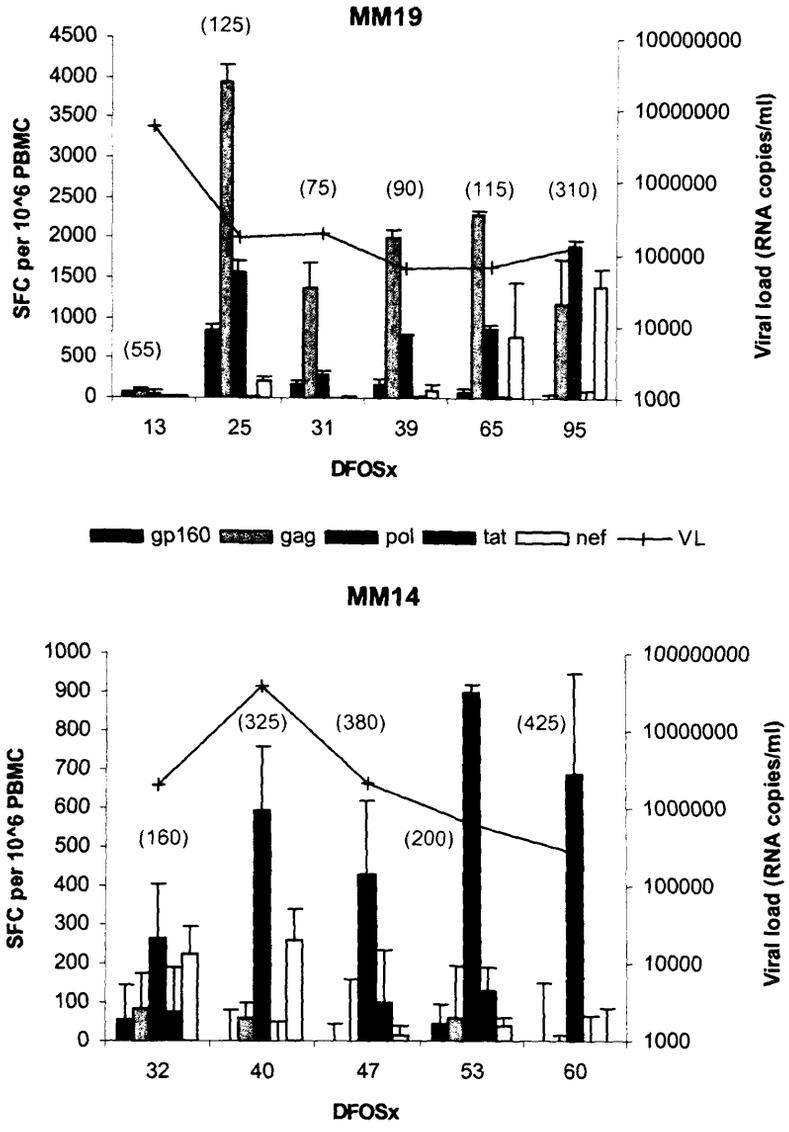


Figure 3.5. Analysis of HIV-specific T cell responses by recombinant vaccinia-stimulated IFN-γ ELISPOT assay during primary infection in HIV-infected individuals who established intermediate-high setpoint viral loads. PBMCs cryopreserved from patients MM9, MM27, MM19 and MM14 at sequential timepoints during infection were stimulated in duplicate or triplicate wells for 48 hours with recombinant vaccinia viruses expressing HIV proteins (gp160, Gag, Pol, Tat and Nef) or a control protein (β-gal), and the number of cells that produced IFN-γ was assessed by ELISPOT assay. Frequencies are expressed as the mean (of duplicate or triplicate wells) number of spot forming cells per 10^6 PBMC after deduction of background values (the number of cells found to produce IFN-γ following stimulation with a recombinant vaccinia virus encoding β-gal only). Figures in brackets indicate the background values (expressed as the number of spot forming cells per 10^6 PBMC) at each timepoint. The error bars indicate 1 SD above the mean. The viral load values shown (HIV RNA copies/ml) were provided by Dr. I. Williams (Mortimer Market Centre, UK).

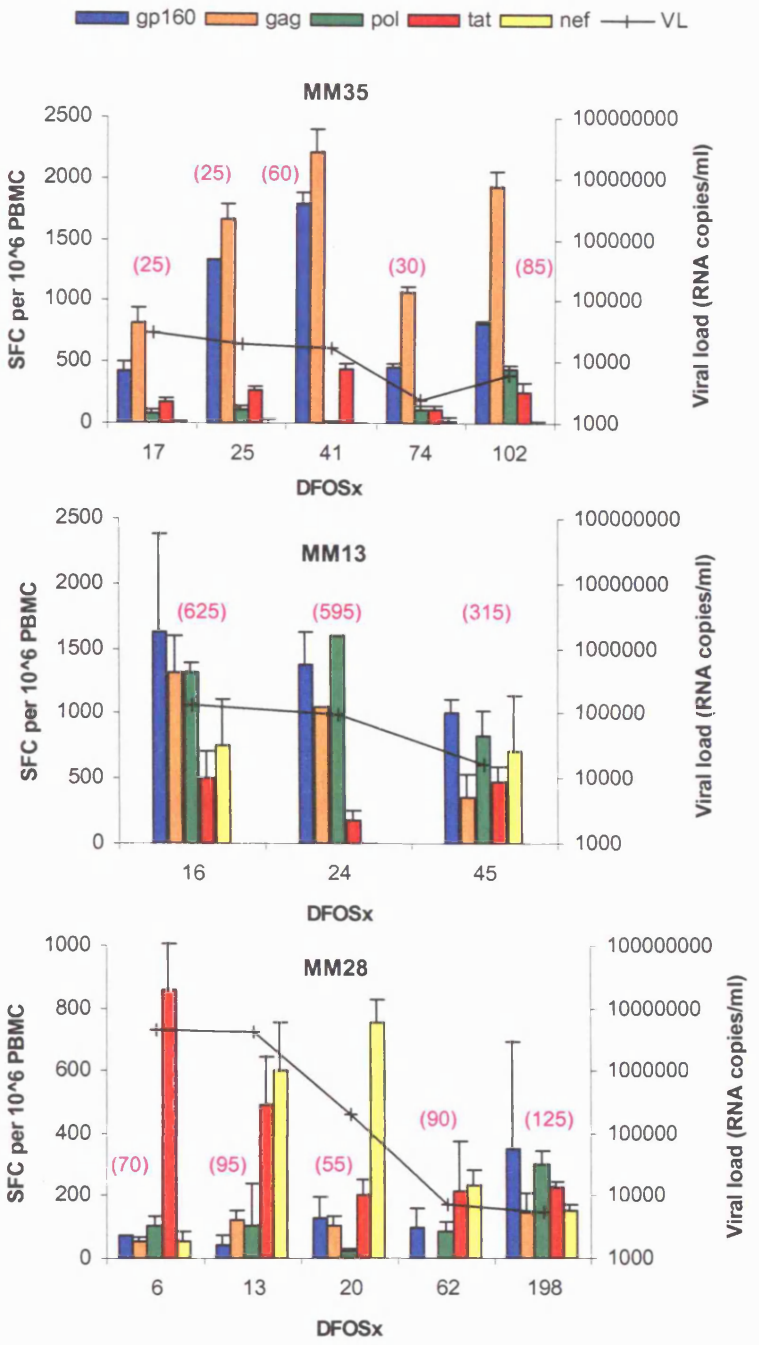


Figure 3.6. Analysis of HIV-specific T cell responses by recombinant vaccinia-stimulated IFN-γ ELISPOT assay during primary infection in HIV-infected individuals who established low-intermediate setpoint viral loads. PBMCs cryopreserved from patients MM35, MM13, and MM28 at sequential timepoints during infection were stimulated in duplicate or triplicate wells for 48 hours with recombinant vaccinia viruses expressing HIV proteins (gp160, Gag, Pol, Tat and Nef) or a control protein (β-gal), and the number of cells that produced IFN-γ was assessed by ELISPOT assay. Frequencies are expressed as the mean (of duplicate or triplicate wells) number of spot forming cells per 10^6 PBMC after deduction of background values (the number of cells found to produce IFN-γ following stimulation with a recombinant vaccinia virus encoding β-gal only). Figures in brackets indicate the background values (expressed as the number of spot forming cells per 10^6 PBMC) at each timepoint. The error bars indicate 1 SD above the mean. The viral load values shown (HIV RNA copies/ml) were provided by Dr. I. Williams (Mortimer Market Centre, UK).

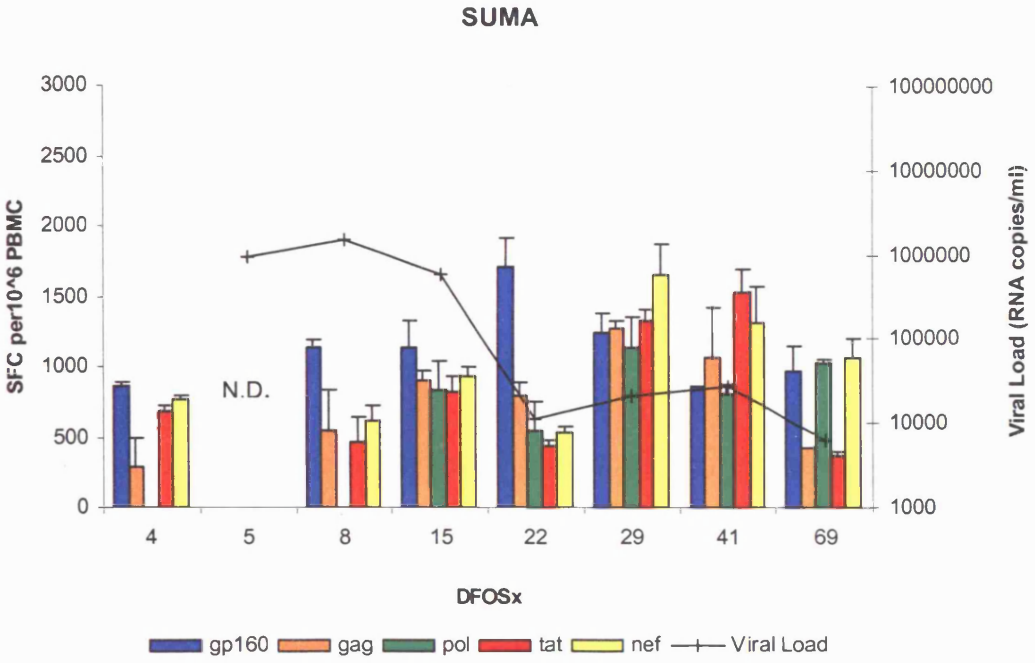


Figure 3.7. Analysis of HIV-specific T cell responses by recombinant vaccinia-stimulated IFN-γ ELISPOT assay during primary infection in an HIV-infected individual who established a low setpoint viral load. PBMCs cryopreserved from patient SUMA at sequential timepoints during infection were stimulated in duplicate or triplicate wells for 48 hours with recombinant vaccinia viruses expressing HIV proteins (gp160, Gag, Pol, Tat and Nef) or a control protein (β-gal), and the number of cells that produced IFN-γ was assessed by ELISPOT assay. Frequencies are expressed as the mean (of duplicate or triplicate wells) number of spot forming cells per 10^6 PBMC after deduction of background values (the number of cells found to produce IFN-γ following stimulation with a recombinant vaccinia virus encoding β-gal only). The error bars indicate 1 SD above the mean. N.D. indicates that PBMC were not available for testing at this timepoint. The viral load values shown (HIV RNA copies/ml) were provided by Dr. G.M. Shaw (University of Alabama at Birmingham, USA).

towards epitope(s) in a limited number (one or two) of the proteins tested. In only two patients (MM13 who established an intermediate-low viral load and patient SUMA who established a low viral load) were broad, co-dominant responses observed to multiple viral proteins from the earliest timepoint tested onwards. In the other patients, the response was more restricted, with fewer viral proteins recognised, and the response to one or two proteins being much more dominant than the others. The proteins to which the response was biased were different for different patients, for example patient MM9 mounted a response very biased towards Pol, but patient MM35 focused his response on gp160 and Gag.

The conclusions that can be drawn from these data are limited. Responses were assessed to only five HIV proteins, which did not correspond to the patients' autologous viral sequences, and responses observed to a given protein could potentially represent recognition of one or many viral epitopes. Nonetheless, these results provided suggestive evidence that there may be some difference in the breadth of the HIV-specific $CD8^+$ T cell response expanded in primary infection in different patients. Further experiments were thus designed to explore this more thoroughly.

3.5 "Comprehensive" epitope mapping using peptide-stimulated IFN-γ ELISPOT assays in patients who controlled early viral replication with differing efficiency

The rVV-stimulated IFN-γ ELISPOT assays initially used gave a limited overview of the HIV-specific $CD8^+$ T cell response mounted during primary infection. To extend the observations made using this assay, I moved on to more comprehensive mapping of the 'entire' HIV-specific $CD8^+$ T cell response in a number of patients, to give more detailed information on the breadth and specificity of the response.

In "comprehensive" mapping experiments, responses made by patient PBMCs to peptides corresponding to the entire HIV-1 clade B consensus sequence were identified. By using peptides corresponding to the consensus sequence, this made mapping the HIV-specific $CD8^+$ T cell response in a large number of patients more feasible than if peptides corresponding to each patient's autologous virus sequence were used. However, it also meant that responses to some epitopes, particularly in more variable regions of the HIV genome, could be missed due to differences between the consensus sequence and autologous sequence.

In total, I used 310 overlapping peptides (20 amino acids in length, overlapping by 10 amino acids) corresponding to the entire HIV-1 clade B consensus sequence (the sequences of which are shown in Table 2.1). To enable fewer cells to be used for mapping and to make the approach more feasible, the 310 peptides were not all tested individually, but were arranged in pools of peptides according to peptide matrices (Table 2.2), and the peptide pools were tested for recognition in an initial IFN-γ ELISPOT assay. The peptides which a patient's cells responded to were deduced from the matrices. A list was then made of the 25-30 peptides most likely to contain an epitope (in accordance with which peptide pools stimulated the largest responses) and these peptides were re-tested individually in a second IFN-γ ELISPOT assay. Although it was possible that a greater number of epitopes could be recognised by a patient, only this number of peptides was chosen for re-testing as the number of cells available was limited. In a similar study by Addo *et al*, only 3 out of 57 patients tested had responses to more than 30 epitopic regions, and all three of these patients were chronically infected with HIV (Addo *et al.*, 2003). Given that responses in primary infection are reported to be narrower than those in chronic infection (Altfeld *et al.*, 2001a; Cao *et al.*, 2003; Dalod *et al.*, 1999b), it was decided that 25-30 peptides was a reasonable number to re-test. In the second assay to confirm which peptides contained epitopic regions, only those which stimulated a response which was greater than three times the background value (the mean of the values of the IFN-γ spot numbers counted in the wells containing cells stimulated with medium alone) were considered to be genuine responses.

To begin with, some preliminary experiments were carried out to verify that this peptide pool-stimulated assay approach was suitable for detecting HIV-specific $CD8^+$ T cell responses. First, to confirm that a response to an individual peptide could be detected when the peptide was included in a pool with other peptides, PBMCs from a patient chronically infected with HIV were screened for responses to the peptide pools containing peptides corresponding to HIV Gag in parallel with all of the individual Gag peptides. The results obtained (Figure 3.8) show that when the peptides were tested individually, peptides 110 and 117 stimulated responses greater than three times the background value, indicating that these peptides contained HIV epitope(s) targeted by this patient's T cell response. From the matrix, it can be seen that if these two peptides did indeed contain epitopes, the patient should

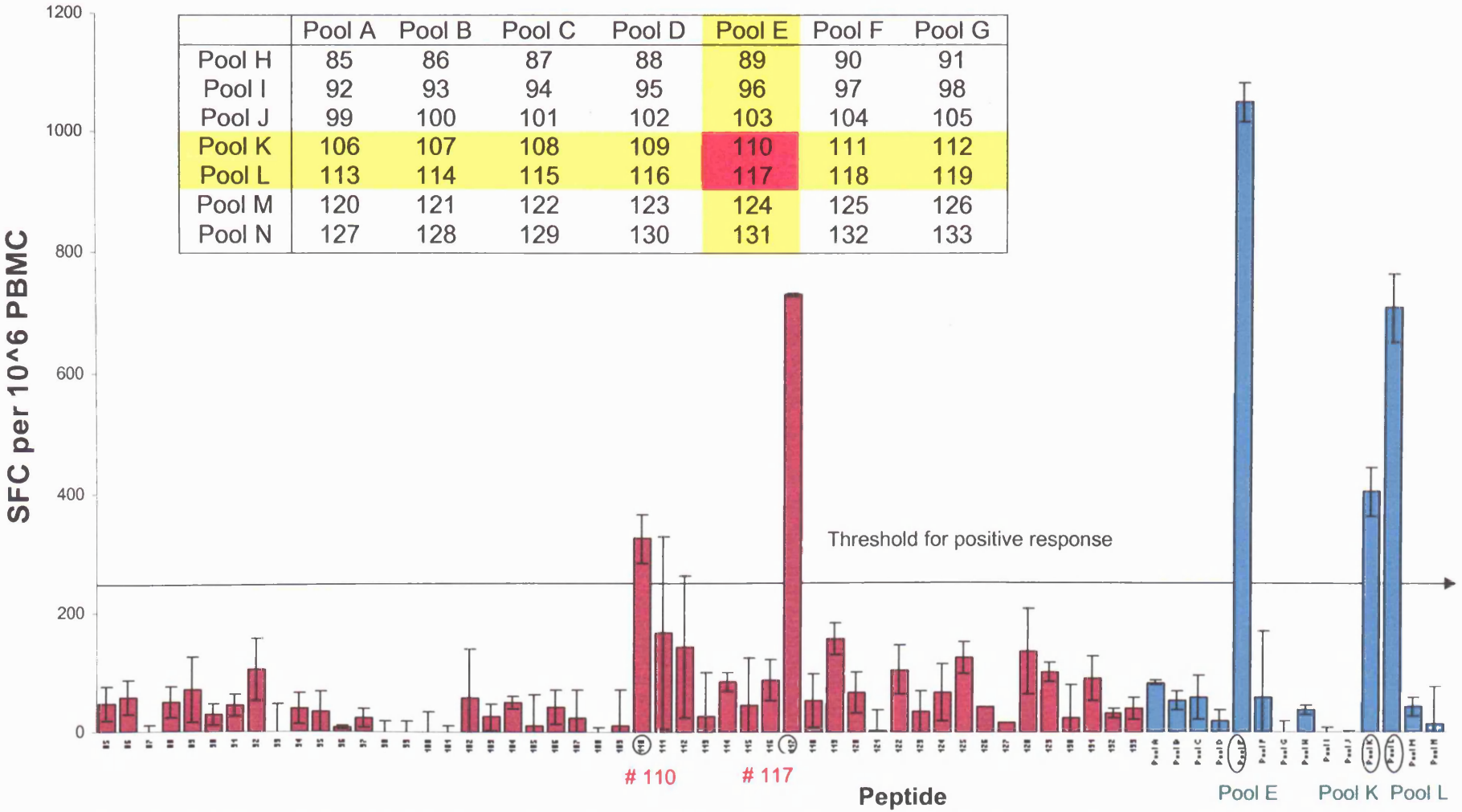


Figure 3.8. Comparison of the use of individual peptides and peptide pools for analysis of HIV-specific responses in IFN-γ ELISPOT assays. 49 overlapping peptides spanning HIV Gag (numbered 85-133) were used either individually or in pools of 7 peptides per pool (A-N) to stimulate PBMC from patient DO (cells taken during chronic infection) in an IFN-γ ELISPOT assay. Responses considered positive were those which stimulated IFN-γ production above the threshold value of three times above the background level, represented by the black arrow (the average number of SFC per 10^6 PBMC produced when cells were stimulated with medium only). The matrix shows how the individual Gag peptides were arranged into pools, and the pools which patient DO responded to are highlighted. From the matrix, it can be seen that recognition of peptides 110 and 117 would give rise to positive responses to these pools.

also respond to peptide pools E, K and L, which was in fact what was observed in the assay. What is more, the magnitudes of the responses made to the peptide pools reflect the magnitudes of the responses observed to the individual constituent peptides: the largest response is to pool E as it is made up of responses to both peptides 110 and 117, and pool L stimulates a larger response than pool K because the individual epitopic region that it contains (peptide 117) stimulates a larger response than the epitopic region that pool K contains (peptide 110). These results show that the epitopic regions to which responses were detected in this patient using the peptide pools would be the same as those that would be detected using individual peptides.

Further experiments were carried out to address whether the responses seen were mediated by $CD8^+$ T cells and to investigate the reproducibility of results between assays.

To investigate the phenotype of the responding cells in peptide-stimulated IFN-γ ELISPOT assays, total PBMC, CD8-depleted PBMC and CD4-depleted PBMC were tested in parallel for responses to peptide pools that a particular patient's cells had previously been shown to exhibit responses to. Figure 3.9 shows the responses made by cells from patient MM11 when three peptide pools from HIV Gag were used to stimulate the three different cell populations. It can be seen that CD8 depletion of the PBMCs resulted in almost a complete loss of the responses to the three peptide pools, compared to just a slight reduction in the magnitude of the response caused by CD4 depletion of the PBMCs (the latter suggesting that cell viability may have been affected to a small extent by depletion). Altogether, this provided strong evidence that the cells making the HIV-specific IFN-γ response in this assay were $CD8^+$ cells.

To investigate the reproducibility of results obtained in peptide pool-stimulated ELISPOT assays, two assays addressing responses to the gp160 peptide matrix were carried out on separate days using different vials of the same patient's cells cryopreserved at the same time, and the same stocks of the peptide pools. As shown in Figure 3.10, the two assays gave very similar results. Interestingly, in these assays, which were carried out with PBMC cryopreserved from a patient during chronic HIV infection, responses were seen towards a high proportion of the gp160 peptide pools, with >65% of the pools tested giving a positive response (three times background) in both assays. Most importantly, the responses seen to the different peptide pools were of a similar hierarchy in the two assays, so the ranked list of long

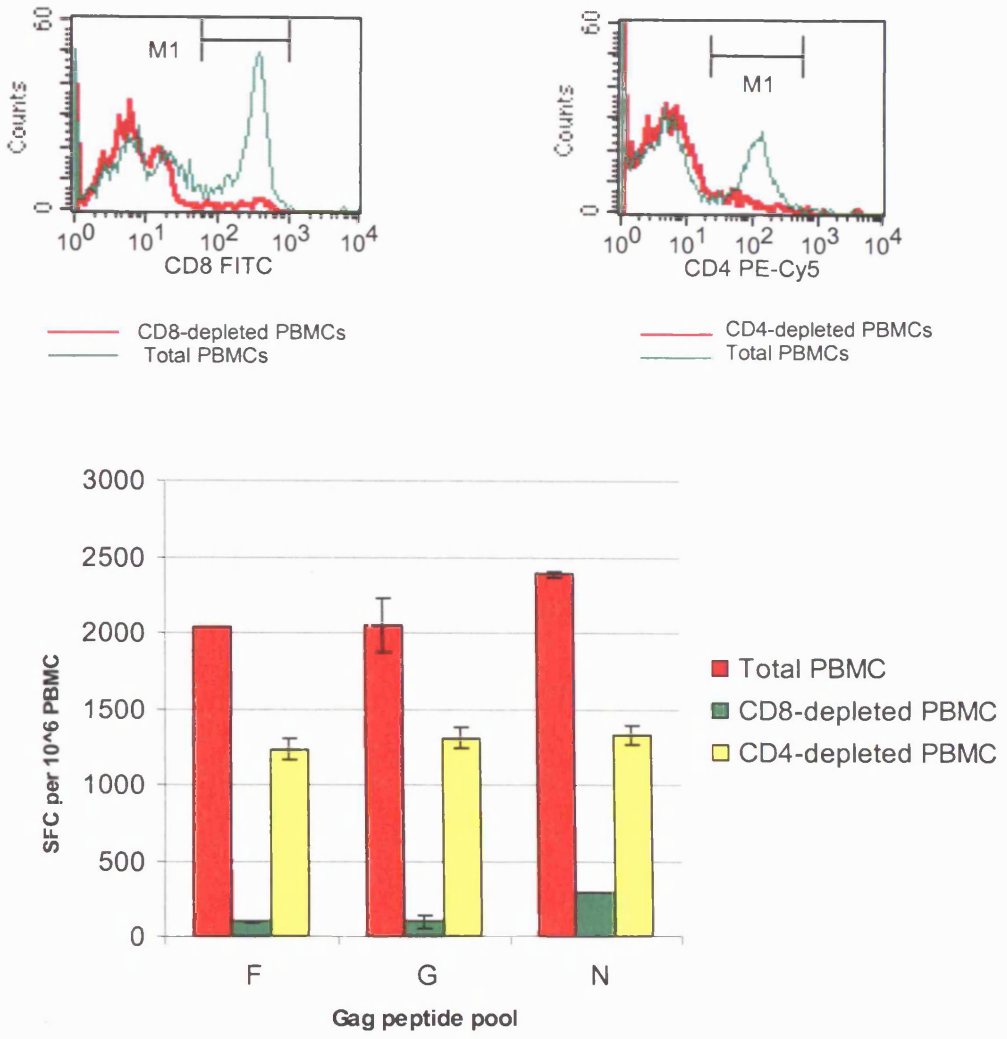


Figure 3.9. Investigation of the phenotype of the T cells mediating the HIV-specific IFN-γ response in peptide pool-stimulated ELISPOT assays. PBMCs cryopreserved from patient MM11 (25 DFOSx) were depleted of either $CD8^+$ or $CD4^+$ cells using magnetic beads. The efficiency of the depletions was assessed by staining total, CD8-depleted and CD4-depleted cells with anti-CD8 FITC and anti CD4 PE-Cy5 monoclonal antibodies. Flow cytometric analysis of the stained cells revealed that the $CD8^+$ cells in the CD8-depleted cell sample had been reduced by 91% and the $CD4^+$ cells in the CD4-depleted cell sample by 78% (top two panels).The three populations of cells were then tested in parallel for responses to three Gag peptide pools that the patient was known to respond to. Cells were stimulated overnight with the peptide pools at a final concentration of 10^{-5}M (or with medium only) and the number of cells stimulated to produce IFN-γ was measured by ELISPOT assay. The results are expressed as the mean (of duplicate wells) number of SFC per 10^6 PBMC after deduction of the background value (mean number of SFC generated when cells were incubated with medium alone). The error bars indicate 1 SD above and below the mean. The background values were 65, 25 and 15 SFC/10^6 PBMC for the total, CD8-depleted and CD4-depleted cells respectively. The results shown are representative of observations made in two independent experiments.

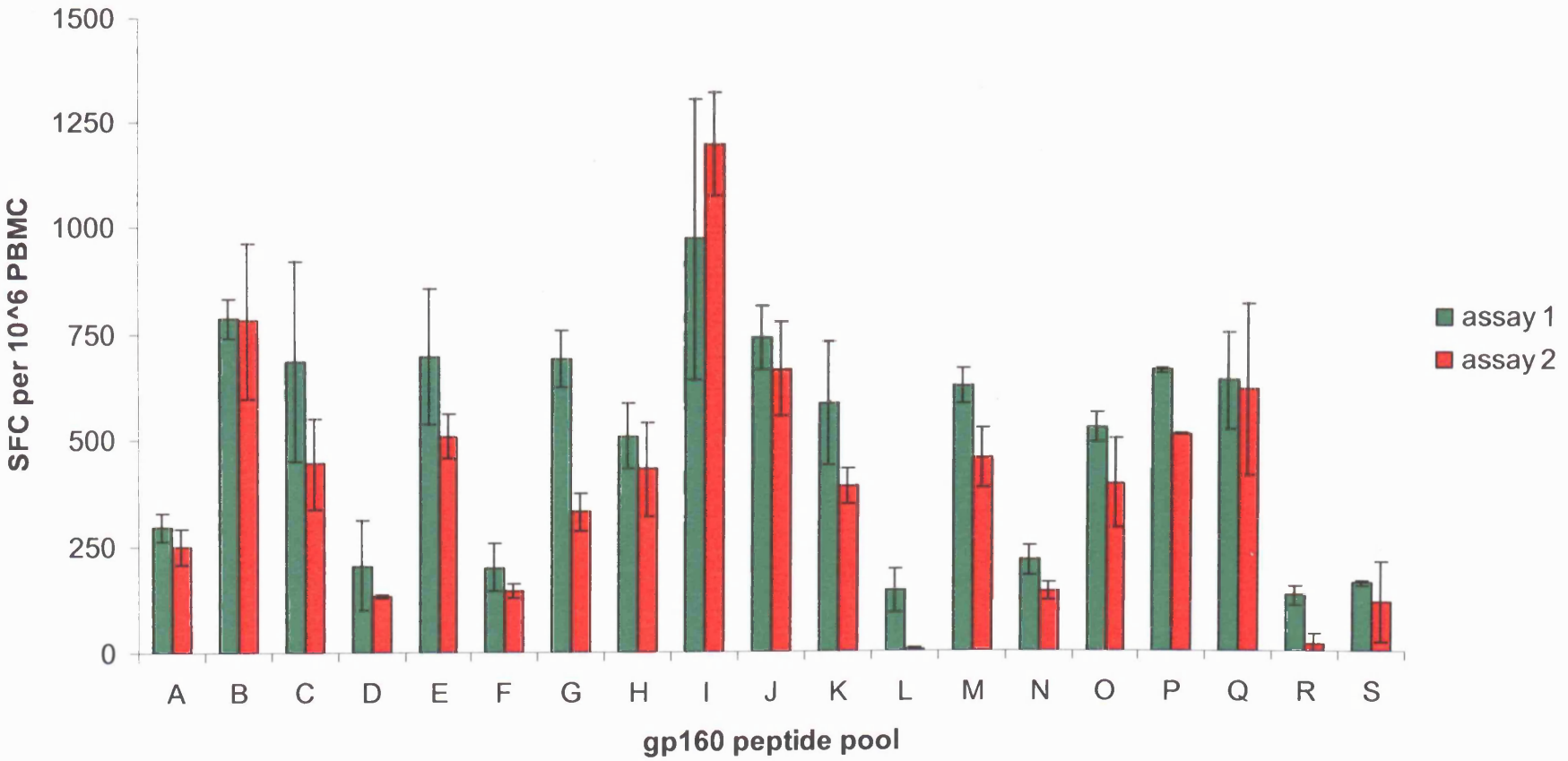


Figure 3.10. Analysis of the extent of assay-to-assay variability between peptide-stimulated IFN-γ ELISPOT assays. PBMC cryopreserved from patient MM4 at 574 DFOSx were stimulated with pools of peptides corresponding to the HIV-1 gp160 clade B consensus sequence at a final peptide concentration of 10^{-5}M. The number of cells stimulated to produce IFN-γ was enumerated by ELISPOT assay and is expressed as the mean (of duplicate or triplicate wells) number of SFC per 10^6 PBMC after deduction of background values (response observed when cells were stimulated with medium alone - the background in assay 1 was 70 SFC per 10^6 PBMC and in assay 2 was 105 SFC per 10^6 PBMC). The red and green bars represent results of two independent experiments using cells cryopreserved from the same patient/timepoints, and the same peptide pool stocks. The error bars indicate 1 SD above and below the mean.

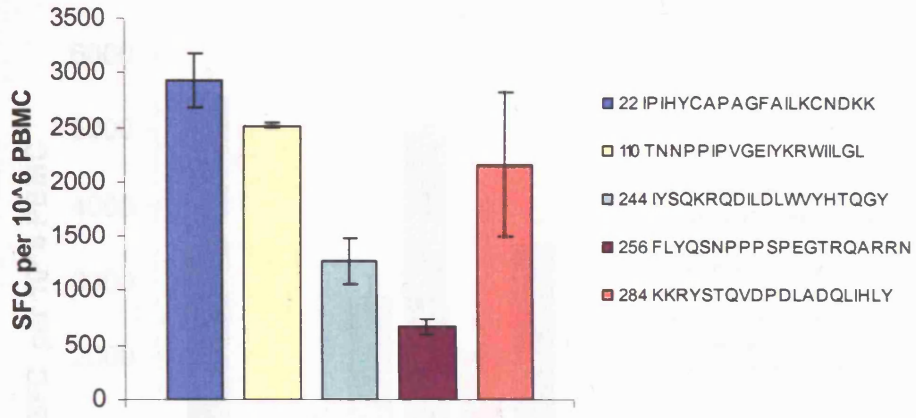
peptides to be re-tested that would be indicated by results from the two replicate assays were very similar (with the peptide pools stimulating the strongest responses being the same in both cases). The variation in the magnitude of responses between replicate wells was also found to be lower in the peptide-stimulated assays than in the rVV-stimulated assays. This could reflect differences in the nature of *in vitro* stimulation of the cells in the two assays, the longer incubation period with a virus possibly having a more detrimental effect on cell survival in the recombinant vaccinia virus-stimulated assay. These results, and similar observations made in duplicate assays using cells from other patients, indicate that the peptide pool-stimulated IFN-γ ELISPOT assays provide a reproducible means of identifying the majority of epitopic regions recognised by the T cell response in a given patient.

Having verified that this approach was suitable for identifying HIV-specific $CD8^+$ T cell responses, this methodology was used to investigate the nature of the early $CD8^+$ response in 13 HIV-1 infected individuals. The first objective was to "comprehensively" identify the epitopic regions recognised by each patient's primary HIV-specific $CD8^+$ T cell response. This was done by initially screening patient cells for responses to the peptide pools, and then re-testing peptides that the initial screen indicated might contain an epitopic region as discussed earlier. All responses were identified using cells cryopreserved at timepoints in early infection (generally between approximately 45 and 100 DFOSx, with the exception of patient SC1 where cells from ~seven months FOSx were used) in IFN-γ ELISPOT assays. The 13 patients studied established different persisting viral loads by ~six months FOSx, falling into three of the four viral load quartiles defined by Mellors *et al* (Mellors *et al.*, 1996): data is shown from five patients who established high viral loads in Figure 3.11, five patients who established intermediate-high viral loads in Figure 3.12, and three patients who established low-intermediate viral loads in Figure 3.13.

Part (i) of each figure summarises the long peptides containing epitopic regions that each patient was found to exhibit a response to, and the relative magnitude of the response to each long peptide. In some cases, responses were observed to two or more overlapping peptides. If responses to two adjacent overlapping peptides were seen, in subsequent calculations of the number of epitopic regions recognised (section 3.6), this was counted as a

(a) Patient MM24

(i) 44-51 DFOSx

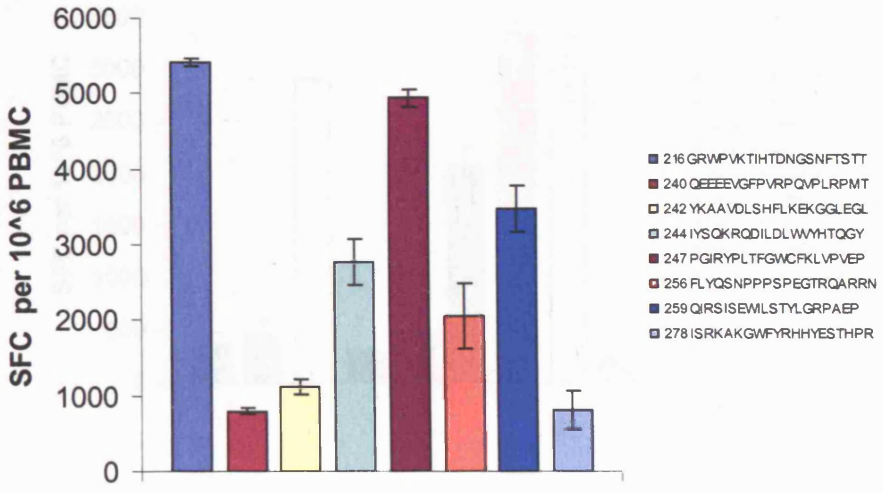


(ii) 211 DFOSx

Long peptide	Epitope peptide	Location	MHC restriction	Magnitude of response (SFC per 10^6 PBMC)
22	CAPAGFAIL	gp160 (aa 216-224)	B51	1309
110	EIYKRWII	gag p24 (aa 128-135)	B8	3320
	(GEIYKRWIIL)		B8	(2424)
	(GEIYKRWII)		B8	(2930)
244	(KRQ)DILDLWVY	nef (aa 105-115)	Cw07	2647 (3812)
244/245	YFPDWQNYT	nef (aa 120-128)	A1	2534
284	LADQLIHLYY	vif (aa 102-111)	A1	379

(b) Patient MM23

(i) 37-113 DFOSx

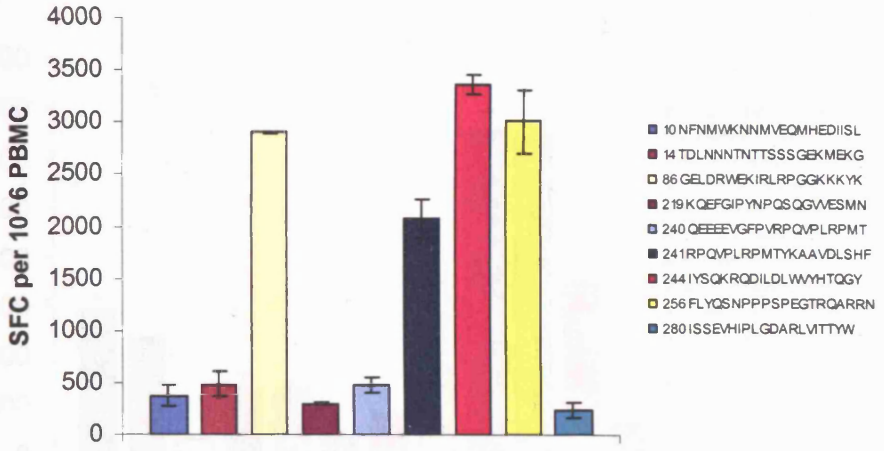


(ii) 113 DFOSx

Long peptide	Epitope peptide	Location	MHC restriction	Magnitude of response (SFC per 10^6 PBMC)
242	FLKEKGGLEGL	nef (aa 90-100)	A2	1952

(c) Patient MM25

(i) 38-94 DFOSx

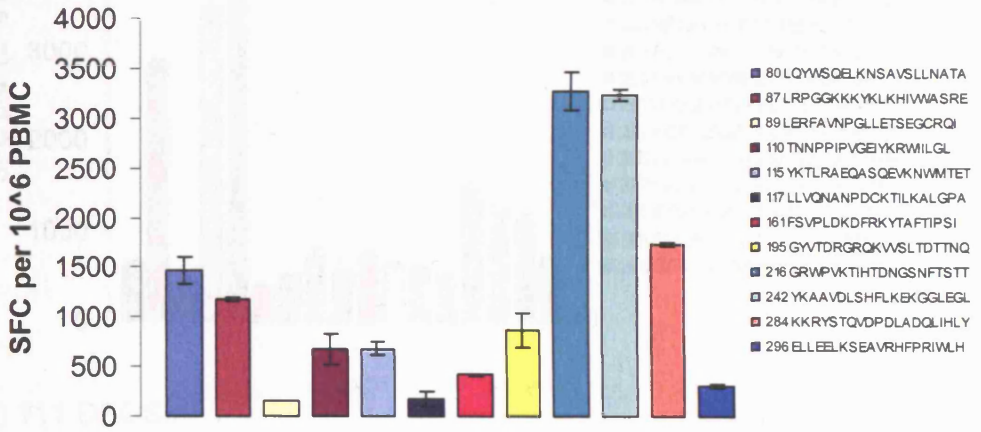


(ii) 94 DFOSx

Long peptide	Epitope peptide	Location	MHC restriction	Magnitude of response (SFC per 10^6 PBMC)
86	RLRPGGKKK(Y)	gag p17 (aa 20-29)	A3	2588 (2291)
241	RPQVPLRPMTY	nef (aa 71-81)	B7	1196
241/242	(A)AVDLSHFLK	nef (aa 83-92)	A3/A11	308 (451)
244	RQDILDLWVY	nef (aa 106-115)	B7	2341
	KRQDILDLWVY	nef (aa 105-115)	Cw07	2731

(d) Patient SC1

(i) ~ 7 months post-infection

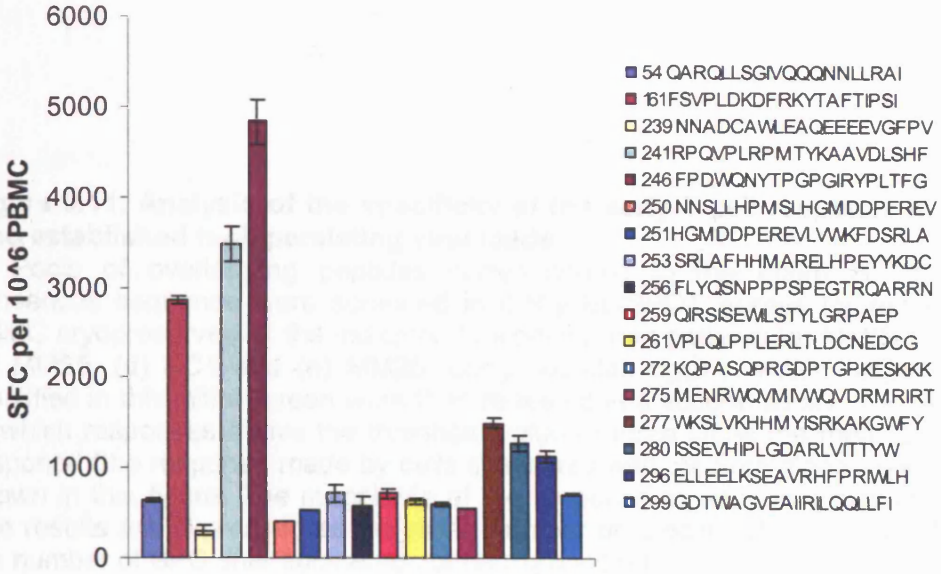


(ii) ~ 10 months post-infection

Long peptide	Epitope peptide	Location	MHC restriction	Magnitude of response (SFC per 10^6 PBMC)
80	ELKSAVSL	gp160 (aa 797-805)	B8	2700
87	GGKKKYKL	gag p17 (aa 24-31)	B8	1253
110	GEIYKRWII	gag p24 (aa 127-135)	B8	883
115	AEQASQEVKNW	gag p24 (aa 174-184)	B44	1070
161	YTAFTIPSI	R.T. (aa 127-135)	A2	1050
216	HTDNGSNF	integrase (aa 114-121)	A1	3079
242	FLKEKGGL	nef (aa 90-97)	B8	3160

(e) Patient MM26

(i) 44 DFOSx



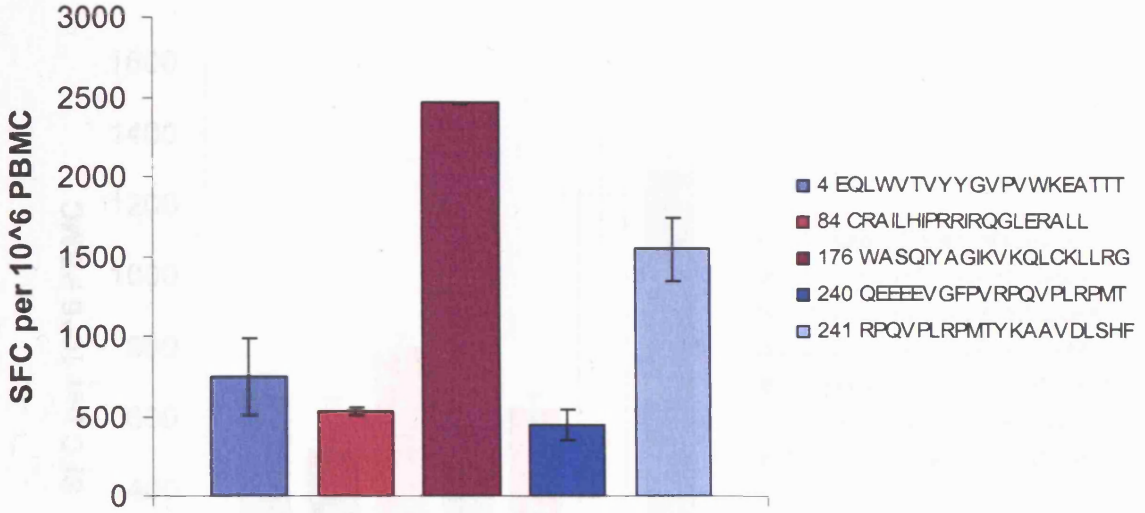
(ii) 111 DFOSx

Long peptide	Epitope peptide	Location	MHC restriction	Magnitude of response (SFC per 10^6 PBMC)
161	(K)YTAFTIPSI	R.T. (aa 126-135)	A2	2367 (2467)
	TAFTIPSI	R.T. (aa 128-135)	A2/B51	2279
241	QVPLRPMTYK	nef (aa 73-82)	A2/B35	2087
	RPQVPLRPMTY	nef (aa 71-81)	B35/B51	1872
	VPLRPMTY	nef (aa 74-81)	B35	1887
246	LTFGWCFKLV	nef (aa 137-146)	A2	1124
246	TPGPGIRYPL	nef (aa 128-137)	B35	2309
253	AFHHMAREL	nef (aa 190-198)	A2	919
277	LVKHHMYISR	vif (aa 24-33)	A68	1059
280	EVHIPLGDAR	vif (aa 54-63)	A68	1004
296	EAVRHFPRI	vpr (aa 29-37)	B51	737
299	RILQQLLFI	vpr (aa 62-70)	A2	962

Figure 3.11. Analysis of the specificity of the early T cell response in patients who established high persisting viral loads.
(i) Pools of overlapping peptides corresponding to the entire HIV-1 clade B consensus sequence were screened in IFN-γ ELISPOT assays for recognition by PBMC cryopreserved at the indicated timepoints from patients (a) MM24, (b) MM23, (c) MM25, (d) SC1 and (e) MM26. Long peptides against which responses were identified in this initial screen were then re-tested in a second assay. Those peptides to which responses above the threshold value of three times the mean background response (the response made by cells stimulated with medium alone) were seen are shown in this figure. The magnitude of the response to each long peptide is given. The results are expressed as the mean number of specific SFC per 10^6 PBMC (i.e. the number of SFC after subtraction of background values), ±1 SD.
(ii) The epitopes recognised within some of the long peptides to which responses were identified in (i) were more precisely mapped by testing short peptides corresponding to epitopes known or predicted to be presented by the patient's HLA alleles within the long peptides for recognition by patient PBMC. The sequences shown are those peptides which stimulated levels of IFN-γ production greater than three times the background response stimulated by medium alone. Where the precise length of the optimal epitope is not known, the additional amino acids of the longer potential epitope are given in brackets. The magnitudes of responses in brackets correspond to the longer peptides which include the additional amino acids. The timepoints at which the cells used for each assay were cryopreserved are indicated in each panel.

(a) Patient MM12

(i) 40-104 DFOSx

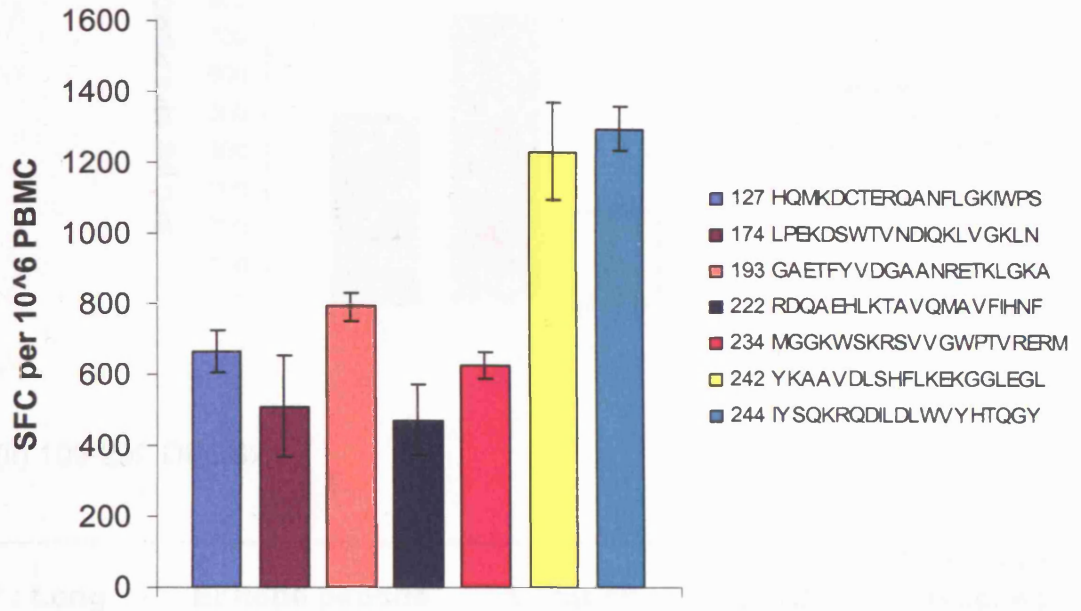


(ii) 230 DFOSx

Long peptide	Epitope peptide	Location	MHC restriction	Magnitude of response (SFC per 10^6 PBMC)
84	IPRRIRQGL	gp160 (aa 834-842)	B7	803
176	QIYAGIKVK	R.T. (aa 269-277)	A3	535
240	FPVRPQVPL(R)	nef (aa 68-77)	B7	578 (633)
241	QVPLRPMTYK	nef (aa 73-82)	A3	1408
	RPQVPLRPMTY	nef (aa 71-81)	B7	1385

(b) Patient MM9

(i) 40-105 DFOSx

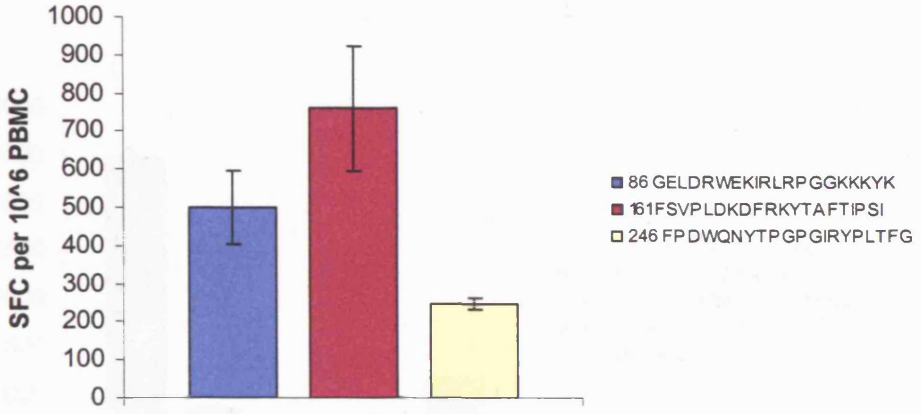


(ii) 273 DFOSx

Long peptide	Epitope peptide	Location	MHC restriction	Magnitude of response (SFC per 10^6 PBMC)
242	FLKEKGGL	nef (aa 90-97)	B8	619

(c) Patient MM27

(i) 53 DFOSx



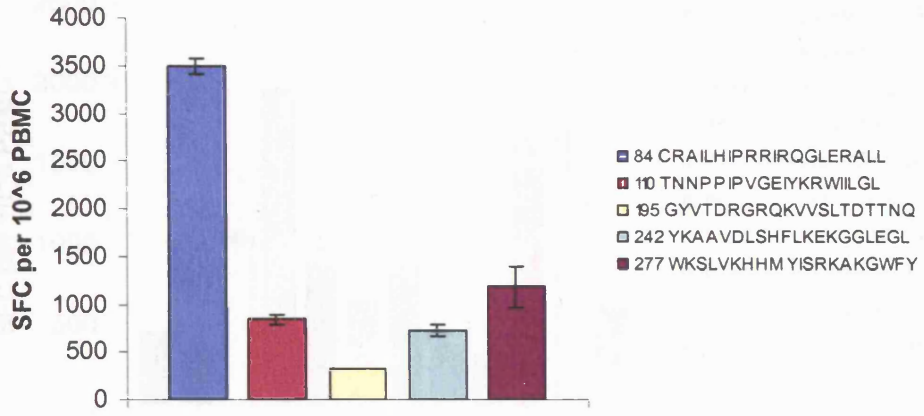
(ii) 109-299 DFOSx

Long peptide	Epitope peptide	Location	MHC restriction	Magnitude of response (SFC per 10^6 PBMC)
86	RPGGKKKYKL	gag p17 (aa 22-31)	B7/A3	347
	(RLRPGGKKK)	gag p17 (aa 20-28)	A3	N.D.
161	(K)YTAFTIPSI	R.T. (aa 126-135)	A2	1457 (1647)
246	TPGPGIRYPL	nef (aa 128-137)	B7	1795

N.D. Not determined by ELISPOT but sequence subsequently confirmed as an epitope using tetramer staining.

(d) Patient MM14

(i) 40 DFOSx

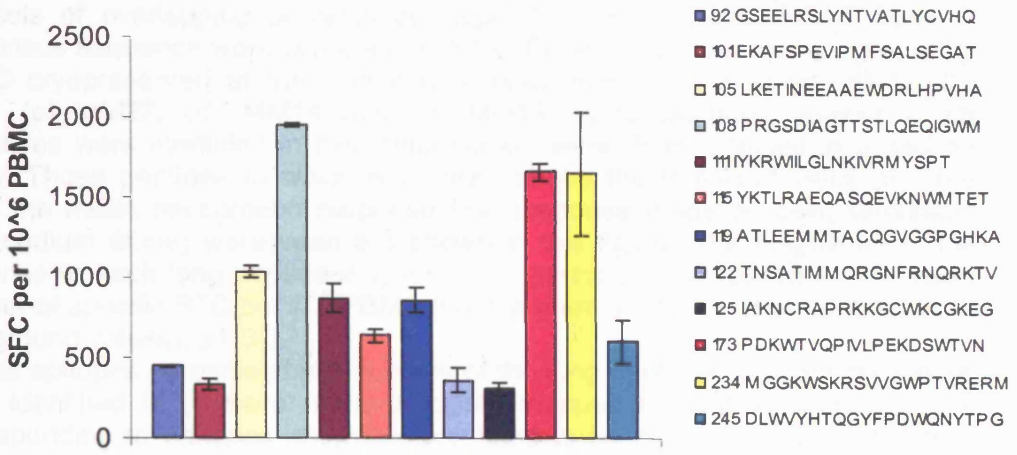


(ii) 111-259 DFOSx

Long peptide	Epitope peptide	Location	MHC restriction	Magnitude of response (SFC per 10^6 PBMC)
84	RRIRQGLERALL	gp160 (aa 836-847)	B8	2108
110	GEIYKRWIIL	gag p24 (aa 127-136)	B8	3101
195	RGRQKVVSL	R.T. (aa 461-469)	B8	1823
242	FLKEKGGL	nef (aa 90-97)	B8	2086
242	AAVDLSHFL	nef (aa 83-91)	A2	686

(e) Patient MM19

(i) 65-102 DFOSx



(ii) 65-102 DFOSx

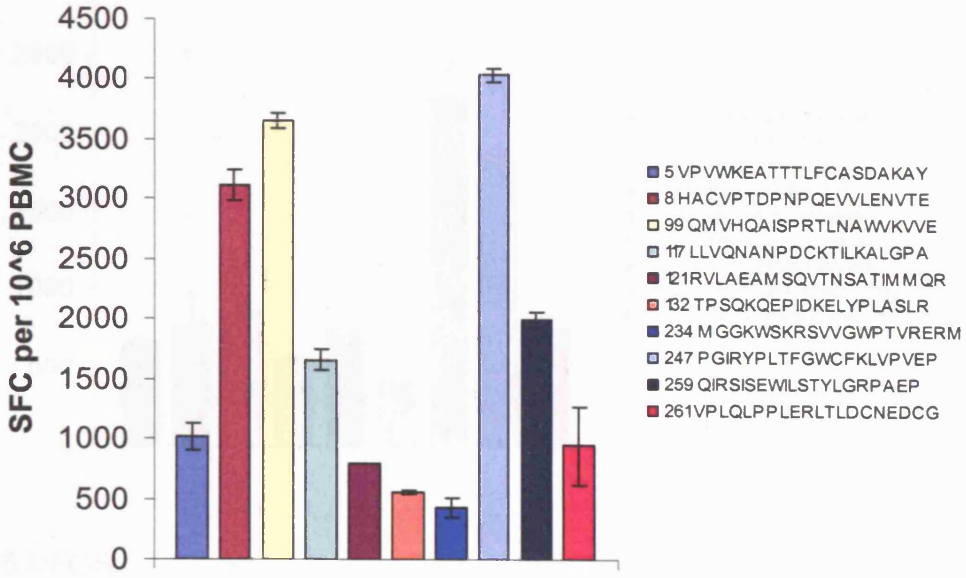
Long peptide	Epitope peptide	Location	MHC restriction	Magnitude of response (SFC per 10^6 PBMC)
92	GSEELRSLY	gag p17 (aa 71-79)	A1	911
101	KAFSPEVIPMF	gag p24 (aa 30-40)	B57	N.D.
108	TSTLQEQIGW	gag p24 (aa 108-117)	B57	N.D.
234	VVGWPTVRER	nef (aa 10-19)	A68	1861

N.D. Not determined by ELISPOT but sequence subsequently confirmed as an epitope using tetramer staining.

Figure 3.12. Analysis of the specificity of the early T cell response in patients who established intermediate - high persisting viral loads.
(i) Pools of overlapping peptides corresponding to the entire HIV-1 clade B consensus sequence were screened in IFN-γ ELISPOT assays for recognition by PBMC cryopreserved at the indicated timepoints from patients (a) MM12, (b) MM9, (c) MM27, (d) MM14 and (e) MM19. Long peptides against which responses were identified in this initial screen were then re-tested in a second assay. Those peptides to which responses above the threshold value of three times the mean background response (the response made by cells stimulated with medium alone) were seen are shown in this figure. The magnitude of the response to each long peptide is given. The results are expressed as the mean number of specific SFC per 10^6 PBMC (i.e. the number of SFC after subtraction of background values), ±1 SD.
(ii) The epitopes recognised within some of the long peptides to which responses were identified in (i) were more precisely mapped by testing short peptides corresponding to epitopes known or predicted to be presented by the patient's HLA alleles within the long peptides for recognition by patient PBMC. The sequences shown are those peptides which stimulated levels of IFN-γ production greater than three times the background response stimulated by medium alone. Where the precise length of the optimal epitope is not known, the additional amino acids of the longer potential epitope are given in brackets. The magnitudes of responses in brackets correspond to the longer peptides which include the additional amino acids.
The timepoints at which the cells used for each assay were cryopreserved are indicated in each panel.

(a) Patient MM35

(i) 45-74 DFOSx

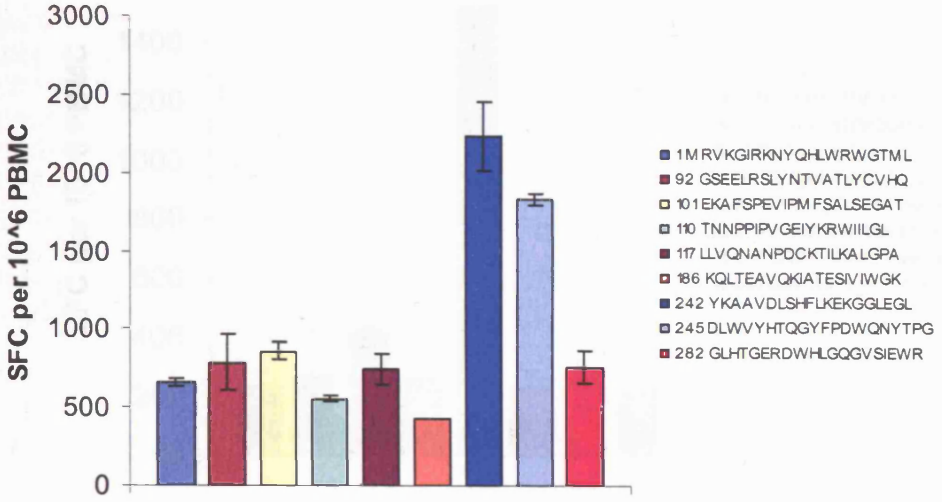


(ii) 192 DFOSx

Long peptide	Epitope peptide	Location	MHC restriction	Magnitude of response (SFC per 10^6 PBMC)
5	LFCASDAKAY	gp160 (aa 51-60)	A24	1078
8	DPNPQEVVL	gp160 (aa 77-85)	B35	1360
117	NPDCKTIL	gag p24 (aa 195-202)	B35	565
246/247	NYTPGPGIRY	nef (aa 126-135)	A24	1203
247	RYPLTFGW(CF)	nef (aa 134-143)	A24	697 (872)
247	YPLTFGWCF	nef (aa 135-143)	B35	502

(b) Patient MM13

(i) 45-98 DFOSx

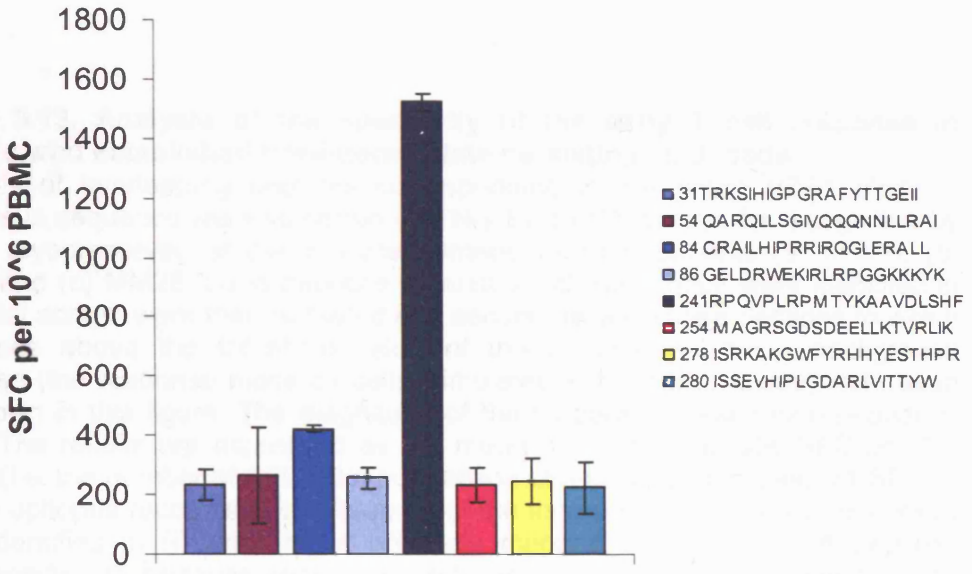


(ii) 275 DFOSx

Long peptide	Epitope peptide	Location	MHC restriction	Magnitude of response (SFC per 10^6 PBMC)
92	GSEELRSLY	gag p17 (aa 71-79)	A1	274
101	KAFSPEVIPMF	gag p24 (aa 30-40)	B57	948
110	(G)EIYKRWII	gag p24 (aa 127-135)	B8	(288) 333
242	FLKEKGGL	nef (aa 90-97)	B8	547
245	HTQYFPDWQ	nef (aa 116-124)	B57	1362
245	YFPDWQNYT	nef (aa 120-128)	A1	204

(c) Patient MM28

(i) 34-93 DFOSx



(ii) 365 DFOSx

Long peptide	Epitope peptide	Location	MHC restriction	Magnitude of response (SFC per 10^6 PBMC)
84	IPRRIRQGL	gp160 (aa 834-842)	B35	399
84	RRIRQGLERALL	gp160 (aa 836-847)	A30	915
86	RLRPGGKKKY	gag p17 (aa 20-29)	A30	893
241	VPLRPMTYK	nef (aa 74-82)	A11	3239
241	RPQVPLRPMTYK	nef (aa 71-82)	B35	2827
241	AAVDLSHFLK	nef (aa 83-92)	A11	442
278	ISRKAKGWFY	vif (aa 31-40)	B35	382

Figure 3.13. Analysis of the specificity of the early T cell response in patients who established low-intermediate persisting viral loads.
(i) Pools of overlapping peptides corresponding to the entire HIV-1 clade B consensus sequence were screened in IFN-γ ELISPOT assays for recognition by PBMC cryopreserved at the indicated timepoints from patients (a) MM35, (b) MM13 and (c) MM28. Long peptides against which responses were identified in this initial screen were then re-tested in a second assay. Those peptides to which responses above the threshold value of three times the mean background response (the response made by cells stimulated with medium alone) were seen are shown in this figure. The magnitude of the response to each long peptide is given. The results are expressed as the mean number of specific SFC per 10^6 PBMC (i.e. the number of SFC after subtraction of background values), ±1 SD.
(ii) The epitopes recognised within some of the long peptides to which responses were identified in (i) were more precisely mapped by testing short peptides corresponding to epitopes known or predicted to be presented by the patient's HLA alleles within the long peptides for recognition by patient PBMC. The sequences shown are those peptides which stimulated levels of IFN-γ production greater than three times the background response stimulated by medium alone. Where the precise length of the optimal epitope is not known, the additional amino acids of the longer potential epitope are given in brackets. The magnitudes of responses in brackets correspond to the longer peptides which include the additional amino acids.
The timepoints at which the cells used for each assay were cryopreserved are indicated in each panel.

response to one epitopic region, since a single epitope could be located in the overlapping region of two adjacent peptides. Further, when calculating the total magnitude of the $CD8^+$ T cell response detected during early infection, only the higher of the responses seen to two adjacent overlapping peptides was included in the final calculation. If responses to three overlapping peptides were seen, the two largest responses were taken. Overall, responses to between 3 (for patient MM27) and 17 (for patient MM26) epitope-containing regions were identified in different patients. The epitope-containing regions identified were located within all HIV protein subunits, with the exception of Protease and Vpu. The magnitude of the total response detected in different patients ranged from around 1,500 up to 21,000 SFC per 10^6 PBMC. A more detailed analysis of the data is presented in section 3.6.

The optimal sequence and the MHC restriction of the epitopes recognised within some of the regions targeted by each patient's T cell response were then further identified. This was done for two purposes: so that tetramers could be obtained (corresponding to one or two immunodominant and subdominant epitopes for selected patients) to enable study of responses to certain epitopes in more detail later on in chapter 5; and to enable the relationship between immunodominance and avidity of T cell responses to be investigated for some patients (in chapter 4). Prediction of optimal epitopes was carried out using information taken from the HIV molecular immunology database (http://www.hiv.lanl.gov/content/hiv-db) on previously described CTL epitopes restricted by the patient's HLA alleles in the long peptide sequences recognised by each patient. If a CTL epitope within a long peptide of interest could not be successfully predicted in this way, then computer algorithms (SYFPEITHI (http://www.syfpeithi.de/) and BIMAS (http://bimas.dcrt.nih.gov/molbio/hla_bind/)) were used to search for all possible 8, 9 or 10mer peptide sequences within the long peptide predicted to bind to any of the patient's HLA alleles. If this gave no suggestions as to potential epitopes, in some cases a more empirical approach was taken and nested sets of peptides were tested for recognition by patient PBMC in an IFN-γ ELISPOT assay, and the optimal epitope deduced from the results. Synthetic peptides corresponding to the optimal epitope peptides predicted or deduced to be recognised by each patient were then tested for recognition by patient cells (not necessarily at timepoints during early infection, but rather where cell availability permitted) to confirm that they were epitopes. As

mentioned before, one aim was to obtain tetramers for the identification of T cells responding to selected epitopes within each patient's response. Since tetramer synthesis is not readily possible for all HLA alleles, for most patients only those epitope peptides which were presented by alleles for which tetramers could be readily obtained were tested. The optimal epitopes to which responses were identified in each patient and their MHC restriction are shown in part (ii) of Figures 3.11-3.13.

3.6 Comparison of features of the early HIV-specific CD8$^+$ T cell response in patients who controlled early viral replication with differing efficiency

The data shown in Figures 3.11 to 3.13 was then analysed in a variety of ways, to give insight into quantitative and qualitative features of the early HIV-specific CD8$^+$ T cell response and whether/how these may differ in patients who subsequently established different persisting viral loads.

However, none of the patients in the London cohort I had available for study established persisting viral loads in the lowest viral load quartile as defined by Mellors *et al* (Mellors *et al.*, 1996). Unfortunately, there were not sufficient cells available from the one patient studied in section 3.4 (SUMA, who was not part of the London cohort) who did establish a very low persisting viral load to enable him to be included in this analysis. This patient's entire primary HIV-specific CD8$^+$ T cell response had already been mapped ((Jones *et al.*, 2004) and Jones *et al* unpublished data), however this analysis was carried out using reagents corresponding to the patient's autologous sequence, rather than the clade B consensus sequence peptides. Since the methods used previously to study SUMA's response were different to those used here, data from this patient could not be included in the inter-group comparisons that follow.

Magnitude

After all epitopic regions recognised by each subject had been identified, the total number of specific spot forming cells per million PBMC to all epitopic regions was calculated as a measure of the magnitude of the entire early response. A wide range in the total magnitude of the response was seen in the different patients, from around 1,500 SFC per 10^6 PBMC up to over 21,000 SFC per 10^6 PBMC. 21,000 SFC per 10^6 PBMC is equivalent to 2.1% of PBMC. Given that CD8$^+$ T cells can constitute 50% of PBMC during primary HIV infection, this equates to up to 4.2% of CD8$^+$ T cells being HIV-specific.

For reasons covered in the discussion, this is likely to be an underestimate of the total percentage of HIV-specific $CD8^+$ T cells in the blood.

In Figure 3.14, the magnitude of each patient's response is plotted against their viral load at the timepoint at which the cells used for this analysis were taken. Looking at the patient group as a whole, there is no relationship between the magnitude of the response and the antigenic load ($p=0.539$).

A comparison of the magnitude of the response in patients grouped according to the persisting viral load they established by ~six months post-infection is shown in Figure 3.15. It can be seen that there was a trend for the patients who established the highest persisting viral loads to have the highest magnitude responses and that this was statistically significant (as revealed by Tukey's pairwise comparison) when the patients with the high and mid-high persisting viral loads were compared ($p<0.05$), but not when those with high and low-mid persisting viral loads were compared ($p>0.05$).

Overall, the majority of patients exhibited very high magnitude HIV-specific $CD8^+$ T cell responses during early infection. The magnitude of the response did not appear to be related to the antigenic load at the time the analysis was carried out; although there was perhaps some tendency for higher magnitude responses to be observed during early infection in patients who went on to establish high persisting viral loads.

Breadth

The number of epitopic regions targeted by the early HIV-specific response was calculated as one measure of the breadth of the response in each subject. In this patient cohort, between 3 and 17 epitopic regions were recognised. Overall, responses were detected during early infection to approximately a fifth of the HIV genome, with 64 out of 310 long peptides being recognised by at least one of the 13 subjects. When the breadth of the response as measured by number of epitopic regions recognised was plotted against the viral load of each subject at the time of analysis (Figure 3.16), there was no correlation between breadth and the level of viraemia in this group of patients ($p=0.359$). There was also no difference in the breadth of the response in patients who subsequently established persisting viral loads in the three different viral load quartiles (Figure 3.17). There was, however, a significant correlation between the number of epitopic regions recognised and the magnitude of the response in the 13 subjects ($p=0.0072$) (Figure 3.18).

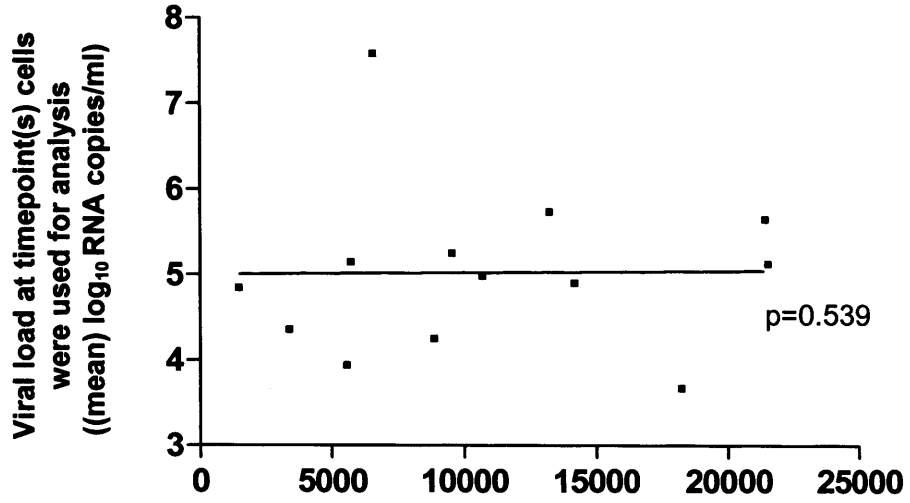


Figure 3.14. Relationship between the viral load and total magnitude of HIV-specific $CD8^+$ T cell responses of patients during early infection. Epitopic regions recognised by the early HIV-specific $CD8^+$ T cell response of 13 subjects were identified as described in the text. The sum of the number of specific spot forming cells per million PBMC raised to all epitopic regions identified as being recognised by a patient's early HIV-specific response was used as a measure of the magnitude of the subject's entire response.
In the graph above, the total magnitude of the response for each patient (in SFC per 10^6 PBMC) is plotted against the viral load of the patient at the timepoint at which cells were taken for analysis (or the mean of the viral loads measured at different timepoints if pooled cells from more than one timepoint were used). A linear regression line is shown, and the p value given was determined using Pearson's correlation test.

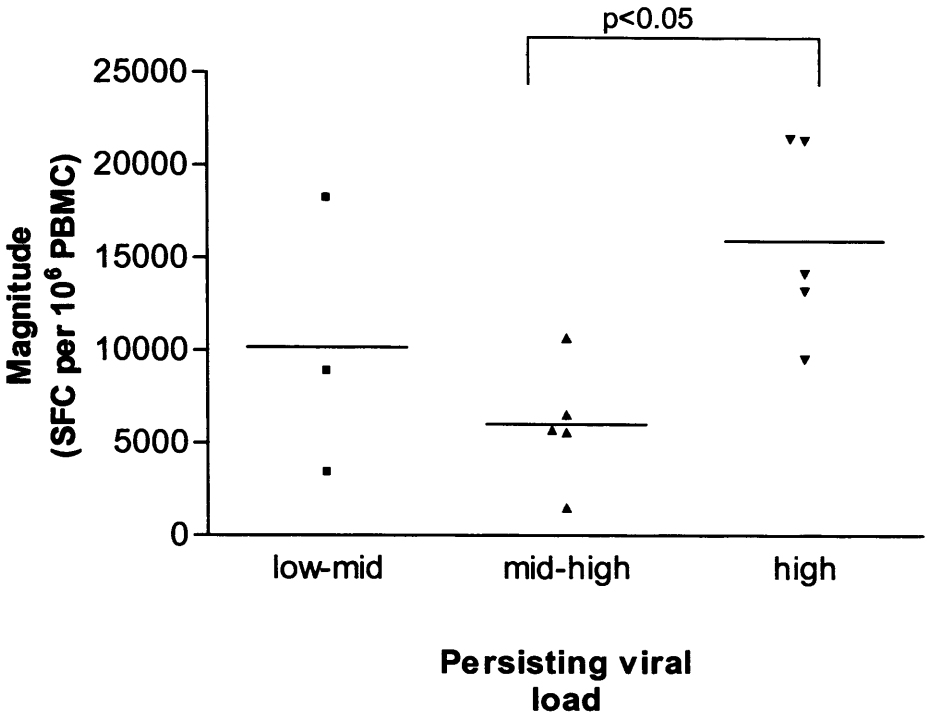


Figure 3.15. Comparison of the total magnitude of the early HIV-specific T cell response detected by IFN-γ ELISPOT assay in patients who established different persisting viral loads. The magnitude of the total HIV-specific $CD8^+$ T cell response during early infection was determined for 13 infected individuals by calculating the total number of specific spot forming cells per million PBMC to all epitopic regions identified as being recognised by their early HIV-specific response (see text).
In the graph above, patients are divided into three groups according to the persisting viral load they established at around 180 DFOSx, as explained in the text: low-mid, mid-high, or high. Each symbol represents the total magnitude of the response detected in early infection in a single patient; the mean values for each group are indicated by the horizontal bars. A one-way ANOVA test with Tukey's multiple comparison post tests were performed to assess whether there was a significant difference between patient groups; only p values which are <0.05 are shown.

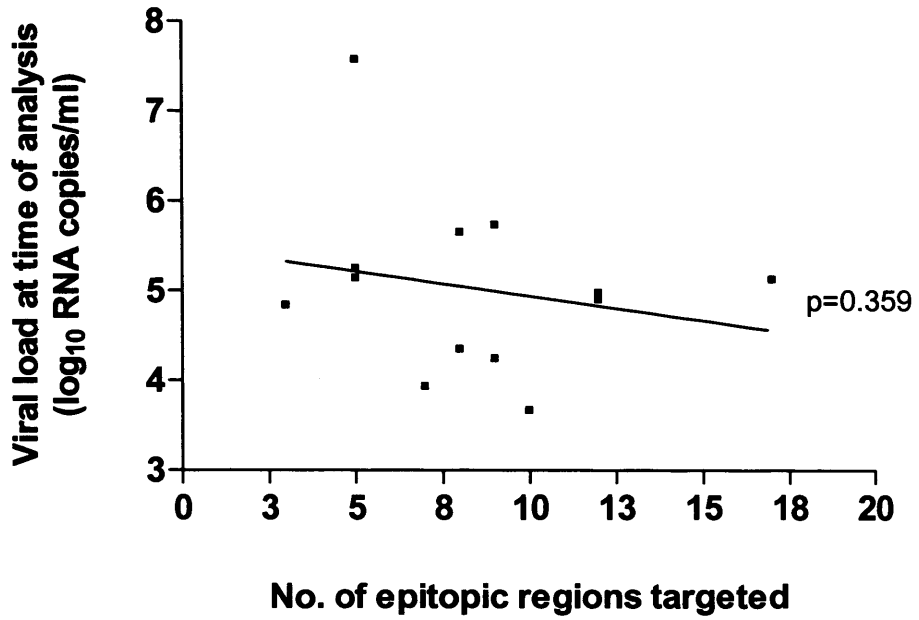


Figure 3.16 Relationship between the viral load at the time of analysis and the breadth of the HIV-specific $CD8^+$ T cell response during early infection in HIV-1 infected subjects. The number of epitopic regions recognised during the early response was determined for 13 infected individuals using IFN-γ ELISPOT assays as described in the text. For each patient, the number of epitopic regions recognised is plotted against the viral load at the timepoint at which cells were taken for analysis (or the mean of the viral loads measured at different timepoints if cells from more than one timepoint were used). A linear regression line is shown, and the p value given was determined using Pearson's correlation test.

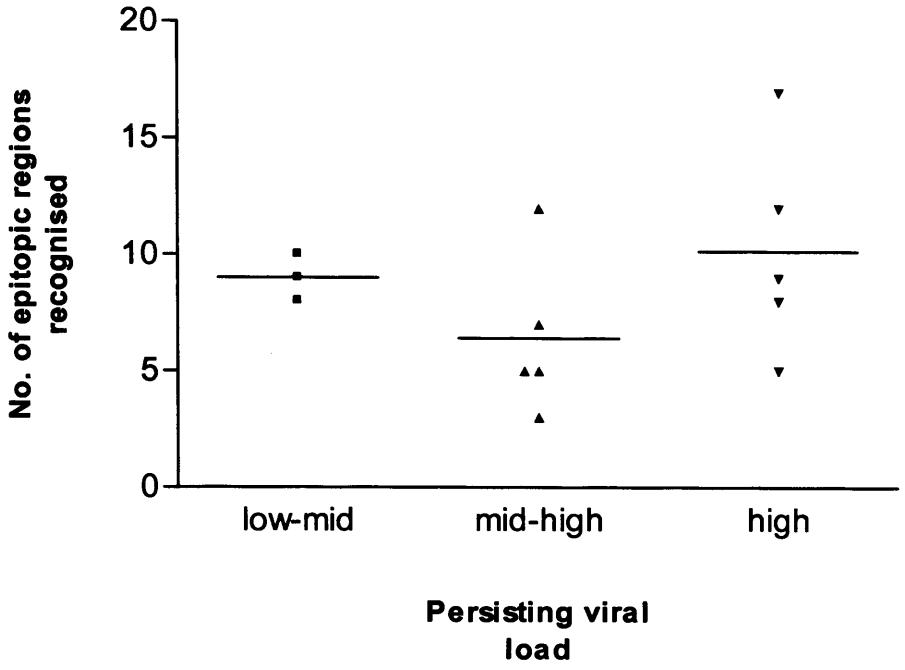


Figure 3.17. Comparison of the number of epitopic regions within the HIV genome targeted by the early T cell response of patients who established different persisting viral loads. The number of epitopic regions recognised during the early response was determined for 13 patients using IFN-γ ELISPOT assays as described in the text. Each symbol represents the number of epitopic regions to which responses were detected during early infection in a single patient. Patients are divided into three groups according to the persisting viral load they established at around 180 DFOSx as explained in the text: low-mid, mid-high or high viral load, and the mean values for each group are indicated by horizontal bars. Statistical analysis (one-way ANOVA) revealed that there was no significant difference in the number of epitopic regions recognised between different patient groups.

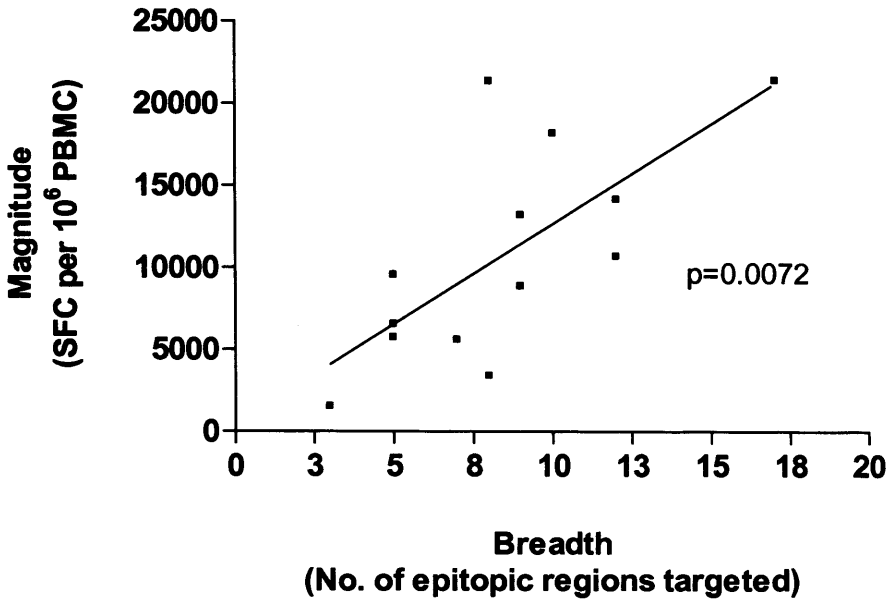


Figure 3.18. Relationship between the total magnitude and breadth of the early HIV-specific $CD8^+$ T cell response. The number of epitopic regions recognised and the total magnitude of the response were determined for 13 infected individuals as explained in the text. The number of epitopic regions recognised is plotted against the magnitude of the response for each patient. A regression line is fitted through the data points and a p value (as calculated using Pearson's correlation test) is given. The p value of 0.0072 indicates that there is a significant positive association between the magnitude and breadth of the early HIV-specific $CD8^+$ T cell response.

Another measure of breadth is the number of protein subunits targeted by the early HIV-specific $CD8^+$ T cell response. The number of protein subunits recognised by a patient was defined as the number of subunits out of a maximum of 14 (gp120, gp41, p17, p24, p2p7p6p1, Protease, Reverse Transcriptase, Integrase, Nef, Rev, Tat, Vif, Vpr and Vpu) in which response(s) to one or more epitopic regions were detected. Between 3 and 8 subunits were found to be recognised in different patients. Analysis of the mean number of protein subunits recognised by groups of patients who established different persisting viral loads revealed that this was highest in the group of patients who established high persisting viral loads, although the difference between the breadth of the response in this patient group was not statistically significant from that in patients who established mid-high or low-mid persisting viral loads at the 0.05 confidence limit (Figure 3.19).

Altogether, these results show that the number of epitopic regions targeted by the CD8 response in early HIV-1 infection in this group of patients was related neither to the antigen load at the time of analysis nor to the persisting viral load established by ~six months post-infection. There was however a tendency for those patients who made large magnitude responses to also target more epitopes.

Specificity

The specificity of the early HIV-specific $CD8^+$ T cell response was explored in a number of ways, to identify immunologically important proteins/protein subunits. In addition to establishing which subunits were recognised, the cumulative magnitude of the response made towards them by all subjects and the proportion of each subject's response targeting specific proteins/protein subunits were also calculated.

Looking at the London patient cohort as a whole, it was observed that most protein subunits could serve as targets for the early HIV-specific immune response, with the detection of responses to one or more epitopic regions within all HIV protein subunits with the exception of Protease and Vpu which were not recognised by any of the 13 subjects.

The frequency of recognition of each protein subunit was determined by calculating the percentage of subjects recognising at least one epitopic region within the subunit. As can be seen in Figure 3.20, the most frequently recognised protein subunit was Nef, with 100% recognition, i.e. all 13 subjects recognised one or more epitopic regions within this protein. Furthermore, the

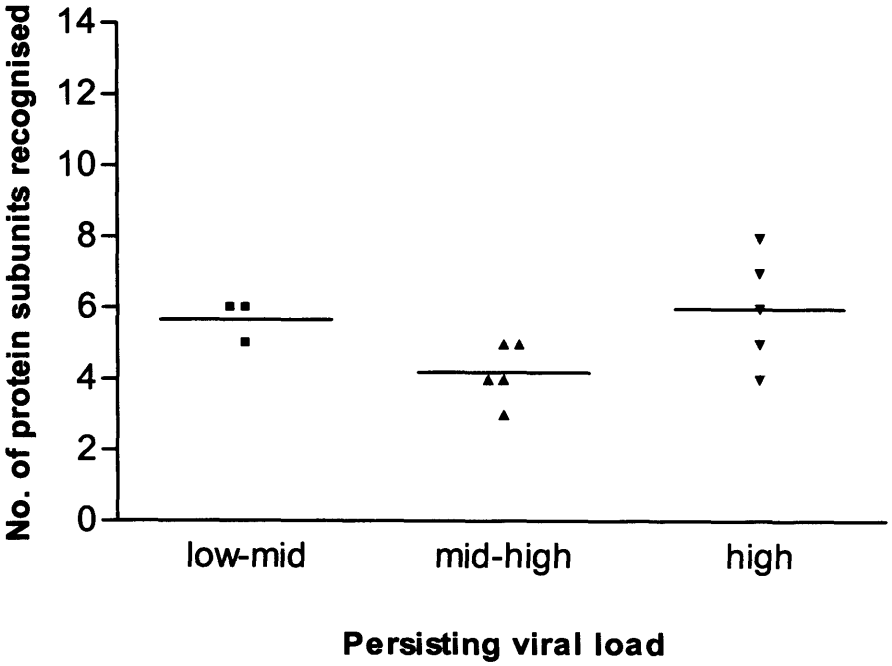


Figure 3.19. Comparison of the number of HIV protein subunits targeted by the early T cell response in patients who established different persisting viral loads. The epitopic regions recognised by the early HIV-specific $CD8^+$ T cell response of each of 13 patients were identified using IFN-γ ELISPOT assays as described in the text. The number of protein subunits recognised (out of a total of 14: gp120, gp41, p17, p24, p2p7p6p1, Protease, Reverse Transcriptase, Integrase, Nef, Rev, Tat, Vif, Vpr and Vpu) was determined for each patient. Recognition of one or more peptides within a protein subunit was counted as recognition of that particular protein subunit. Each symbol represents the number of protein subunits recognised in early infection by a single patient. Patients are divided into three groups according to the persisting viral load they established at around 180 DFOSx as explained in the text, and the mean values for each group are indicated by horizontal bars. Statistical analysis (one-way ANOVA) revealed that there was no significant difference in the number of protein subunits recognised between different patient groups.

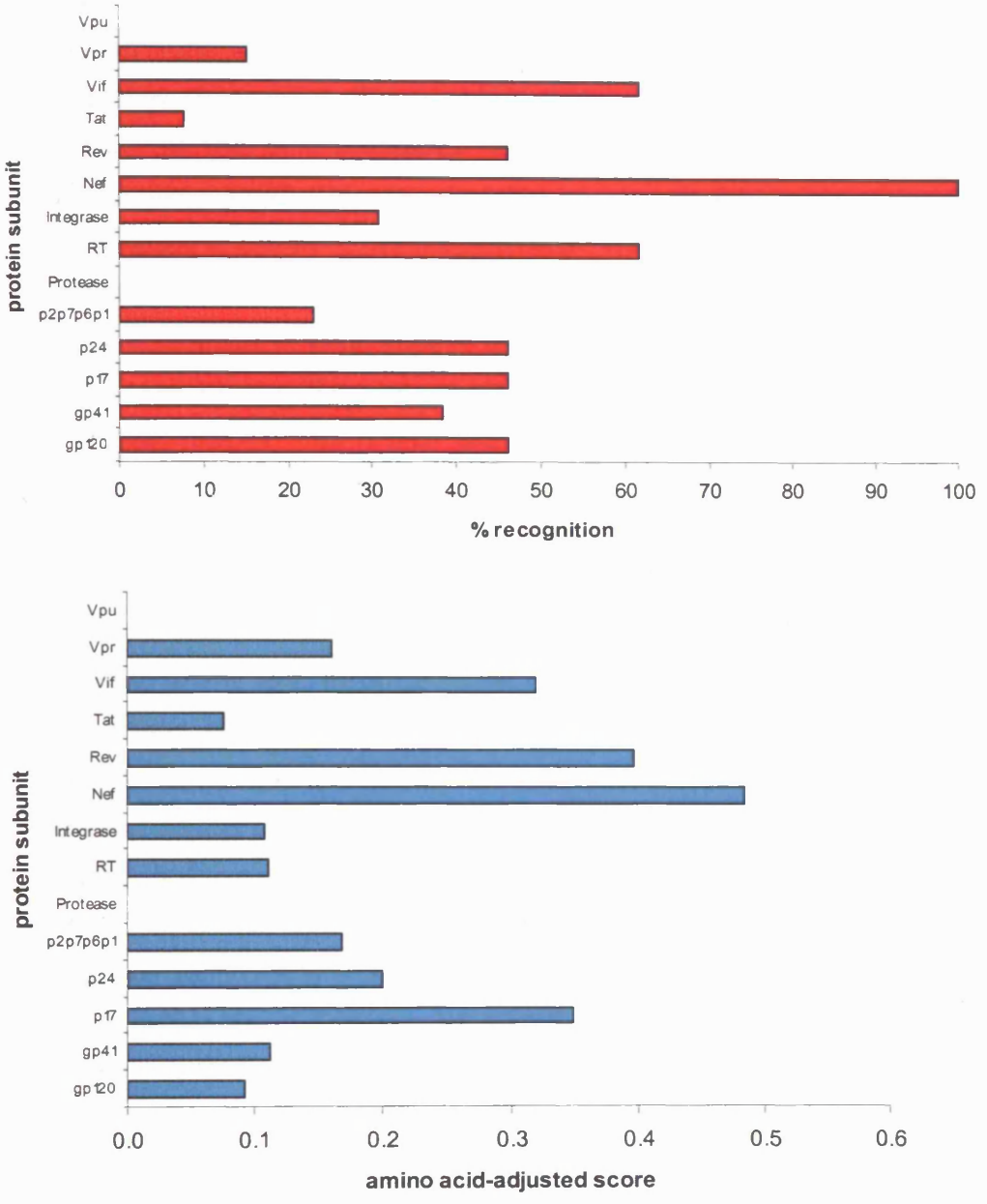


Figure 3.20. Frequency of recognition of individual HIV-1 protein subunits. The epitopic regions within the HIV-1 proteome recognised during early infection by PBMC from 13 HIV-infected individuals were determined as described in the text. The frequency of recognition of each protein subunit by the patient cohort was determined by calculating the percentage of patients with a response to at least one epitopic region within the subunit. The amino acid-adjusted score represents the frequency of recognition of each subunit divided by the subunit length in amino acids.

most frequently targeted long peptide (YKAAVDLSHFLKEKGGLEGL) was located in Nef, with 6 out of 13 patients responding to it. The next most recognised proteins were RT and Vif. However when the results were adjusted to take into consideration the size of the subunits, although Nef was still the most immunogenic subunit, in this case Rev and Gag p17 were the next most frequently recognised (Figure 3.20). A similar analysis was carried out to explore the size of the response targeted at different proteins, since even a quantitatively small response would be counted as recognition of a subunit in the analysis of protein subunit recognition in Figure 3.20, which hence may not reflect the true immunogenicity of the protein subunits. The total magnitudes of responses made to each subunit were calculated by finding the total number of SFC per 10^6 PBMCs raised to each subunit in all patients responding to that particular subunit. The results are shown in Figure 3.21, where the protein subunits have been ranked in order of the magnitude of the responses they stimulate. Taking into account the size of the subunits, the distribution of SFC frequencies per amino acid length was also determined (Figure 3.21). Looking at the amino-acid adjusted scores, overall the greatest response was to epitopes within Nef, followed by Rev. As well as being the most frequently recognised subunit, Nef was therefore also the subunit against which the highest total response was targeted in this patient cohort.

Another aspect of the specificity of the early response of interest was whether it is related to the persisting viral load established later. To address this, for each subject the proportion of their entire early HIV-specific CD8 response targeting each of four major viral protein regions (Env, Gag, Pol and Nef) (i.e. the total number of SFC per 10^6 PBMC directed towards all epitopic regions recognised within a certain protein expressed as a percentage of the total magnitude (SFC per 10^6 PBMC) of the patient's early response) was plotted against the persisting viral load as shown in Figure 3.22. Statistical analysis revealed that there was no association between the total proportion of the response directed towards the protein and the persisting viral load for any of the proteins studied in this way ($p=0.914$, 0.473, 0.844 and 0.585 for Env, Gag, Pol and Nef respectively), i.e. there was no evidence to suggest that there is preferential targeting of a specific protein by patients with higher or lower viral loads.

It has been shown that $CD8^+$ T cell responses which target epitopes within proteins that are expressed early during the viral lifecycle (such as Nef, Tat and Rev) control viral replication *in vitro* more effectively than responses which

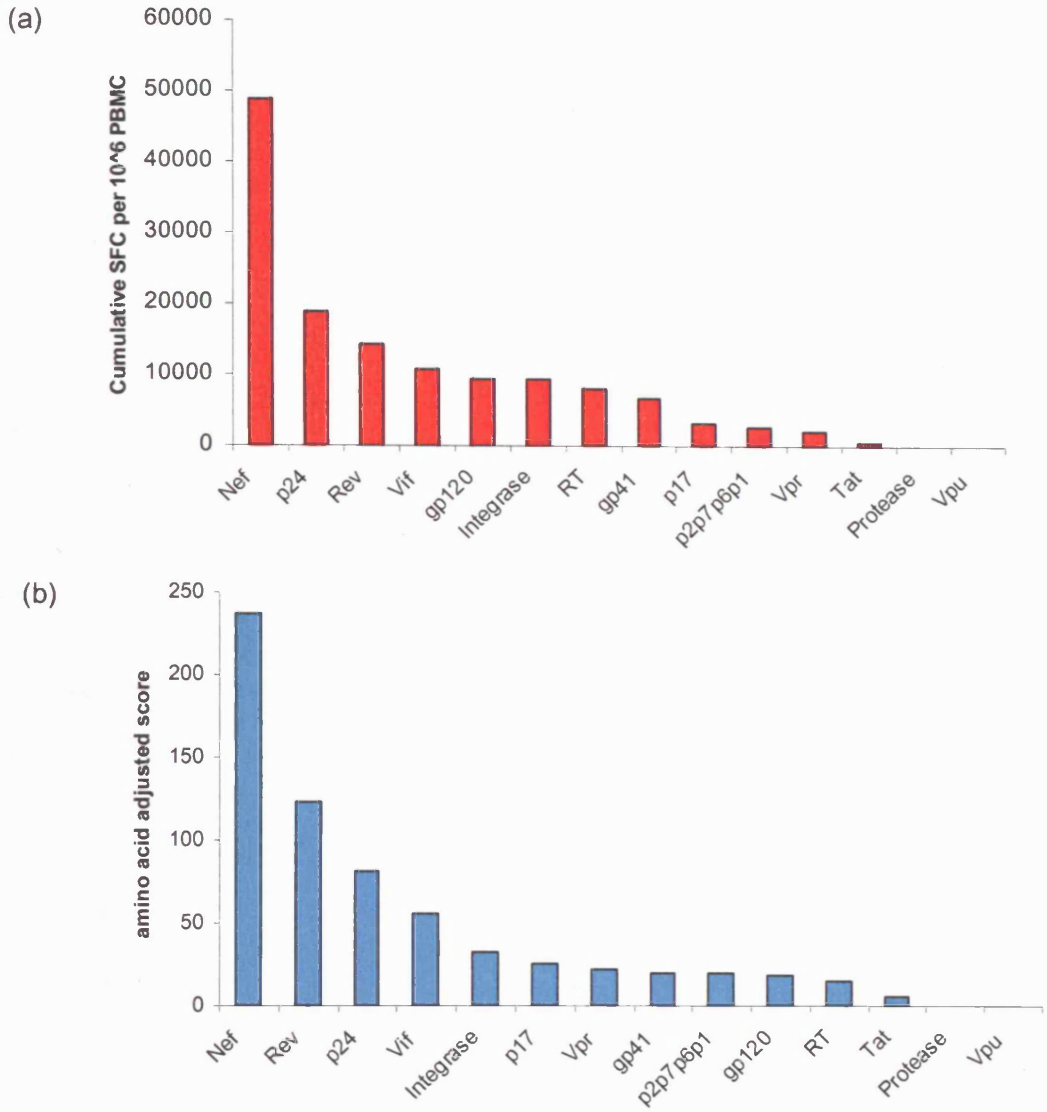


Figure 3.21. Total magnitudes of responses detected to HIV-1 protein subunits in IFN-γ ELISPOT assays in 13 infected subjects. The specificity and magnitude of the early HIV-specific $CD8^+$ T cell response in 13 subjects were determined as described in the text. The total magnitude of responses made to each HIV protein subunit was determined by calculating the total SFC per 10^6 PBMC made by the study group to all peptides corresponding to the subunit as shown in chart (a).The different sizes of the protein subunits were taken into account in (b) by dividing the total magnitude as indicated in (a) by the length of the subunit. Both the total magnitude of the responses made to each subunit and the amino acid-adjusted scores are represented in the graphs in a hierarchy from highest to lowest recognition.

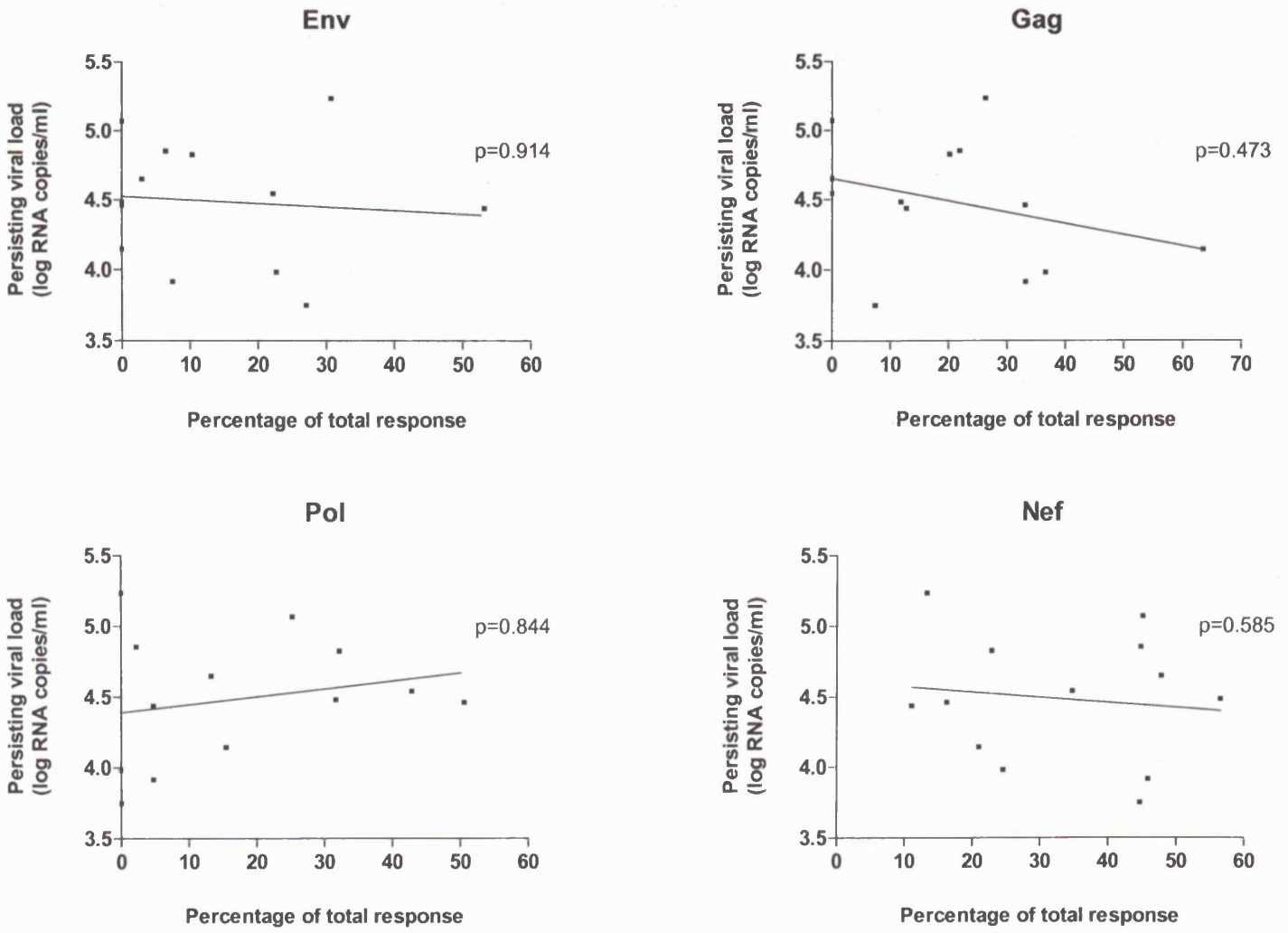


Figure 3.22. Relationship between specificity of the early HIV-specific CD8$^+$ T cell response and the persisting viral load established by HIV-infected subjects. The proportion of the early HIV-specific CD8$^+$ T cell response directed against HIV Env, Gag, Pol and Nef was determined for each of 13 HIV-1 infected individuals by calculating the total magnitude of responses to all peptides corresponding to the HIV protein as a proportion of the total HIV-specific response (the total magnitude of the response raised to all epitopic regions found to be targeted by the patient's early response, determined as explained in the text). Each point represents the proportion of the early response specific for a particular HIV protein in one individual and is plotted against the persisting viral load they established by ~180 DFOSx. Linear regression lines are shown in each panel; the p values given were determined using Pearson's correlation tests.

target epitopes within proteins which are expressed later in the lifecycle (van Baalen *et al.*, 2002; Yang *et al.*, 2003). The proportion of the response targeting early proteins was determined for each patient by calculating the sum of the magnitude of all responses made to epitopic regions within Nef, Tat and Rev as a percentage of the total magnitude of all the responses made by the subject. This ranged from 11% of the response in one patient up to as much as 70% of the response in another patient. To see if there was any association between responses mounted towards epitopes within early viral proteins and a more favourable disease course, the proportion of the response targeting early proteins was compared for patients within different viral load groups. In Figure 3.23 it can be seen that the early-protein specific response was quite variable for patients within one group, and that there was no evidence that early proteins were targeted any more or less by patients with different disease prognoses ($p>0.05$ for all inter-group comparisons).

Biasing of the response

Earlier in this chapter, results from rVV-stimulated ELISPOT assays addressing the response to five HIV proteins suggested that in the majority of the subjects the response towards these proteins was unevenly distributed, with skewing of the response towards one or two proteins in many cases. To confirm and expand upon this observation, analysis was carried out using data from the comprehensive epitope mapping studies to see if there was biasing of the response towards certain regions of the HIV genome firstly at the protein level, and secondly, at the epitope level.

The percentage of the response directed towards each protein was calculated (i.e. the sum of the responses to epitopic regions recognised by a patient within a given protein divided by the total magnitude of the response, multiplied by 100), and the two most immunodominant proteins identified. The sum of the magnitudes of the responses towards these two proteins was calculated as a percentage of the magnitude of the total response for each patient and is shown in Figure 3.24, where patients have been divided up into three groups depending on the persisting viral load established by ~six months FOSx. In all 13 patients, greater than 55% of the total early response was directed against just two viral proteins, confirming earlier observations made using rVV-stimulated ELISPOT assays that the primary HIV-specific $CD8^+$ T cell response is biased towards a small number of immunodominant proteins. The extent of biasing was greatest in patients who established mid-high

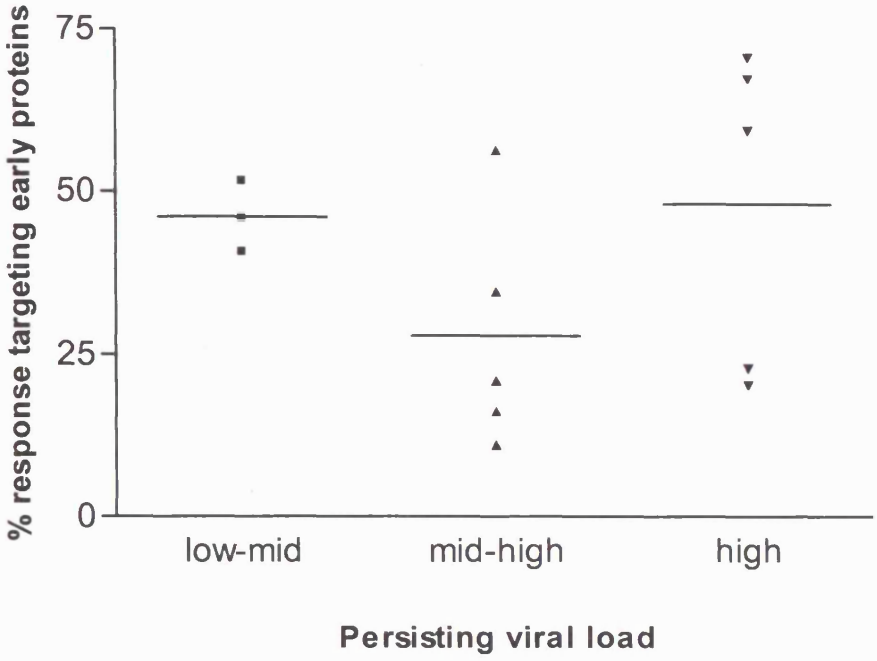


Figure 3.23. Comparison of the proportion of the early HIV-specific T cell response targeted against proteins expressed early in the viral life cycle in HIV-infected individuals who established different persisting viral loads. The epitopic regions recognised by the early $CD8^+$ T cell response in 13 patients and the magnitude of the response to each region were identified as described in the text. The percentage of the early HIV-specific T cell response directed towards proteins expressed early in the viral life cycle (Rev, Tat and Nef) was determined for each individual by calculating the sum of the magnitude of the responses directed towards epitopic regions in Rev, Tat and Nef as a percentage of the total magnitude of the response. Each symbol represents the percentage of the early response targeting early proteins in a single patient. Patients are divided into three groups according to the persisting viral load they established at around 180 DFOSx (as explained in the text): low-mid, mid-high or high viral load, and the mean values for each group are indicated by horizontal bars. Statistical analysis (one-way ANOVA) revealed that there was no significant difference in the in proportion of the response targeted against proteins expressed early in the viral life cycle between different patient groups.

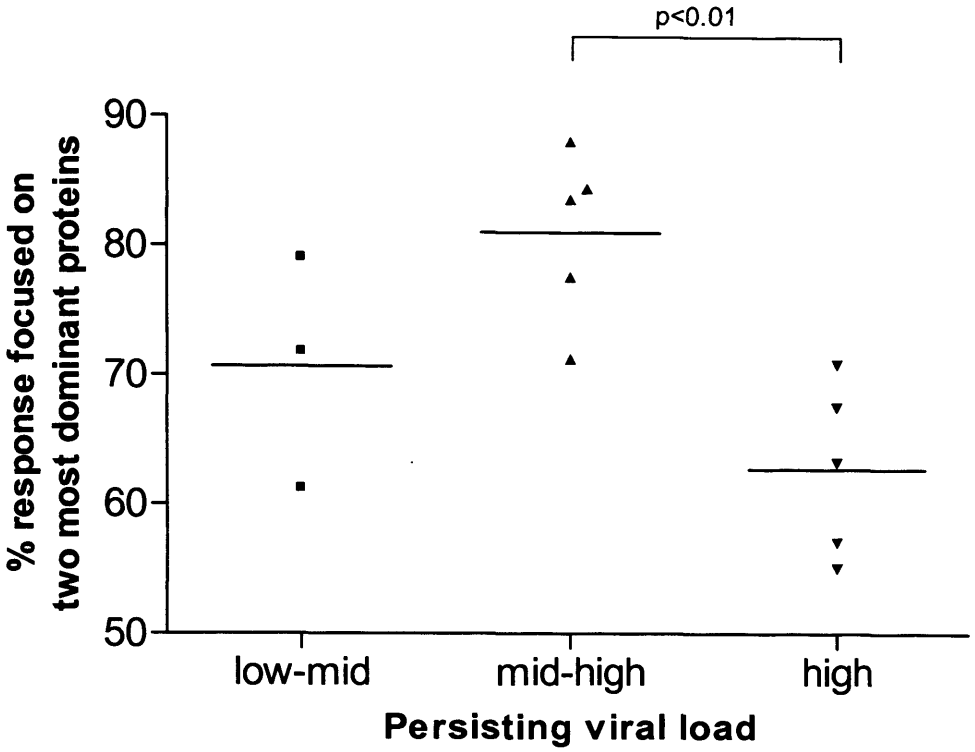


Figure 3.24. Comparison of the extent of biasing of the early HIV-specific T cell response towards the two most immunodominant proteins in patients who established different persisting viral loads. The epitopic regions within the HIV-1 proteome recognised by the early HIV-specific $CD8^+$ T cell response (and the magnitude of the response to each region) were identified for each of 13 patients as described in the text. The two proteins to which the highest percentages of the total response were directed were identified for each individual, and the percentage of the early HIV-specific T cell response directed towards these two proteins was calculated by expressing the sum of the magnitude of the response to them as a percentage of the total magnitude of the patient's response. Each symbol represents the percentage of the response focused on the two most dominant proteins during early infection in a single patient. Patients are divided into three groups according to the persisting viral load they established at around 180 DFOSx (as explained in the text): low-mid, mid-high or high viral load. The mean values for each group are indicated by horizontal bars. A one-way ANOVA test with Tukey's multiple comparison post tests were performed to assess whether there was a significant difference between patient groups; only those p values resulting from inter-group comparisons which are <0.05 are shown.

persisting viral loads (and statistical analysis revealed that this was significantly greater compared to those who established high persisting viral loads ($p<0.01$), although the reasons for this are unclear).

In order to study the extent of biasing of the response at the epitope level, the four most dominant epitopes (i.e. the four which elicited the largest responses) within the response of each patient were determined and the sum of the magnitudes of the responses towards them were calculated as a percentage of the total magnitude of the patient's response. In Figure 3.25 it can be seen that for all patients, greater than 50% of their entire response was targeted towards the most immunodominant epitopes. Hence at the epitope level, there was also a high degree of biasing of the response towards a small number of immunodominant regions of the proteome in all patients studied. Comparing patients in different viral load quartiles, although the extent of biasing of the response towards the most dominant epitopes appeared to be greater for the patients with mid-high and high than that for patients with low-mid persisting viral loads, this was not significantly significant ($p>0.05$ for all inter-group comparisons).

These results confirmed that the early HIV-specific T cell response in most patients is highly biased towards a small number of viral proteins (an observation made earlier using rVV-stimulated ELISPOT assays) and a small number of epitopic regions within the viral proteome. Earlier results suggested that there may potentially be somewhat less bias in the response of those patients establishing the lowest persisting viral loads; here no patients establishing low persisting viral loads were available for study, so whether their response differed in the extent of biasing from that of other patients could not be addressed.

3.7 Analysis of TCR Vβ usage by HIV epitope-specific T cells in patients who established different persisting viral loads

In the previous section, the breadth of the HIV-specific $CD8^+$ T cell response in different patients was described in terms of either the number of HIV proteins or the number of epitopic regions the patient recognised in IFN-γ ELISPOT assays. Another aspect of the breadth of a response is the number of different clones of T cells involved – the clonal breadth. T cell clones can be distinguished on the basis of their TCR use. The TCR is a membrane bound heterodimer made up of an α and a β chain. Each chain is encoded by a series of variable (V), diversity (D), joining (J) and constant (C) segments. The

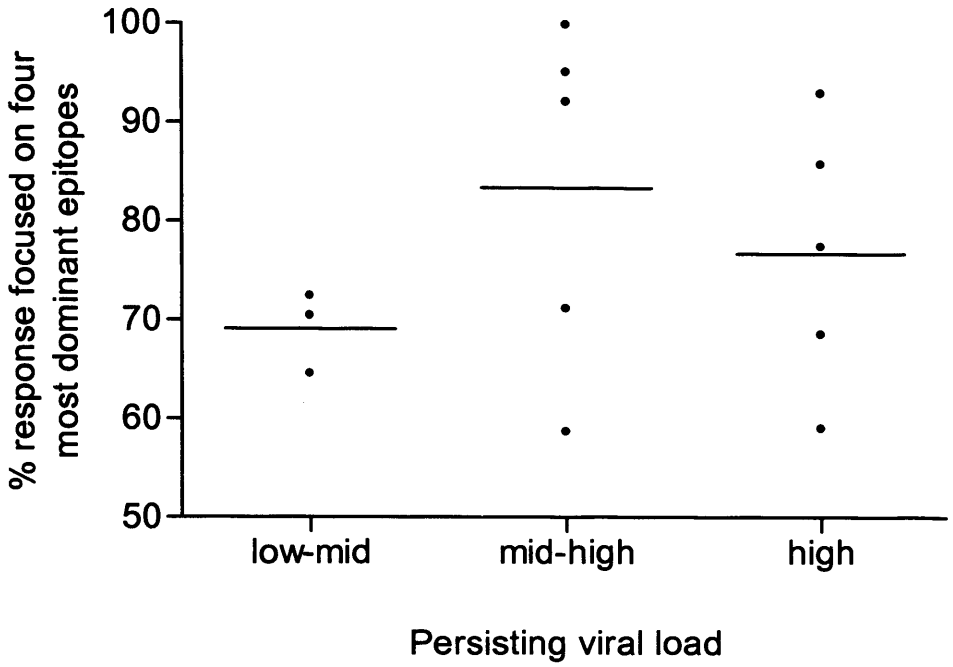


Figure 3.25. Comparison of the extent of biasing of the early HIV-specific T cell response towards the four most dominant epitopic regions in patients who established different persisting viral loads. The epitopic regions recognised by the early HIV-specific $CD8^+$ T cell response (and the magnitude of the response to each region) were identified for each of 13 patients as described in the text. The four epitopic regions towards which the highest magnitude responses were directed were determined for each patient, and the percentage of the early HIV-specific T cell response directed towards these four epitopic regions was calculated by expressing the sum of the magnitude of the response to them as a percentage of the total magnitude of the patient's response. Each symbol represents the percentage of the response focused on the four most dominant epitopes during early infection in a single patient. Patients are divided into three groups according to the persisting viral load they established at around 180 DFOSx (as explained in the text): low-mid, mid-high or high viral load. The mean values for each group are indicated by horizontal bars. Statistical analysis (one-way ANOVA test) revealed that there was no significant difference in the extent of biasing of the response towards the four most dominant epitopic regions between different patient groups.

broad diversity of the TCR repertoire results from the numerous possible combinations of V, D and J segments, from random mutations or nucleotide additions and from the random pairing of α and β chains. There are a variety of techniques for assessing the repertoire of T cell clones involved in an immune response, including TCR heteroduplex analysis and CDR3 length spectratyping. I chose to assess the pattern of Vβ family usage by T cells responding to individual HIV epitopes in different patients by co-staining patient PBMCs with tetrameric peptide-MHC complexes (details of which are given in chapter 5) and antibodies specific for different TCR Vβ families. Although this does not allow quantitation of the number of individual T cell clones involved in a response (as within a given Vβ family, more than one clone of cells may be involved), it gives an overview of the diversity of TCR Vβ families used in a given response, allowing this to be compared between responses in different patients.

In experiments described in chapter 5, tetrameric peptide-MHC complexes were obtained to allow identification of T cells responding to dominant and subdominant epitopes in different patients, and the kinetics of expansion of epitope-specific responses were characterised. Here, the tetramers were used to analyse Vβ family usage by T cells responding to a dominant epitope in four patients (one patient in each of the four different viral load quartiles) at the peak of the epitope-specific response (determined in chapter 5). The epitopes and patients studied were as follows: A3 RLRPGGKKK in MM25, a patient who established a high persisting viral load; A3 QIYAGIKVK in MM12, a patient who established an intermediate-high persisting viral load; B8 FLKEKGGL in MM13, a patient who established a low-intermediate persisting viral load and B15 MTKGLGISY in SUMA, a patient who established a low persisting viral load. PBMC taken from a timepoint at or close to the peak of each response were then co-stained with a HIV tetramer, antibodies to CD3 and CD8, plus one of a panel of Vβ family-specific antibodies. Flow cytometric analysis of samples revealed the percentage of tetramer positive cells using each Vβ family, the results of which are shown in Figure 3.26. The A3 RLRPGGKKK -specific $CD8^+$ T cells in MM25's response used only 3 out of 22 Vβ families tested, with a strong bias towards the use of Vβ 2 (~50% tetramer positive cells using Vβ 2). By comparison the A3 QIYAGIKVK-specific CD8 cells in patient MM12 used a broader range of Vβ families (10/22), with a slight bias towards Vβ 2 and Vβ 5.3. Although the B8 FLKEKGGL-specific response in MM13 used a moderate number of Vβ families (6/22), there was particularly

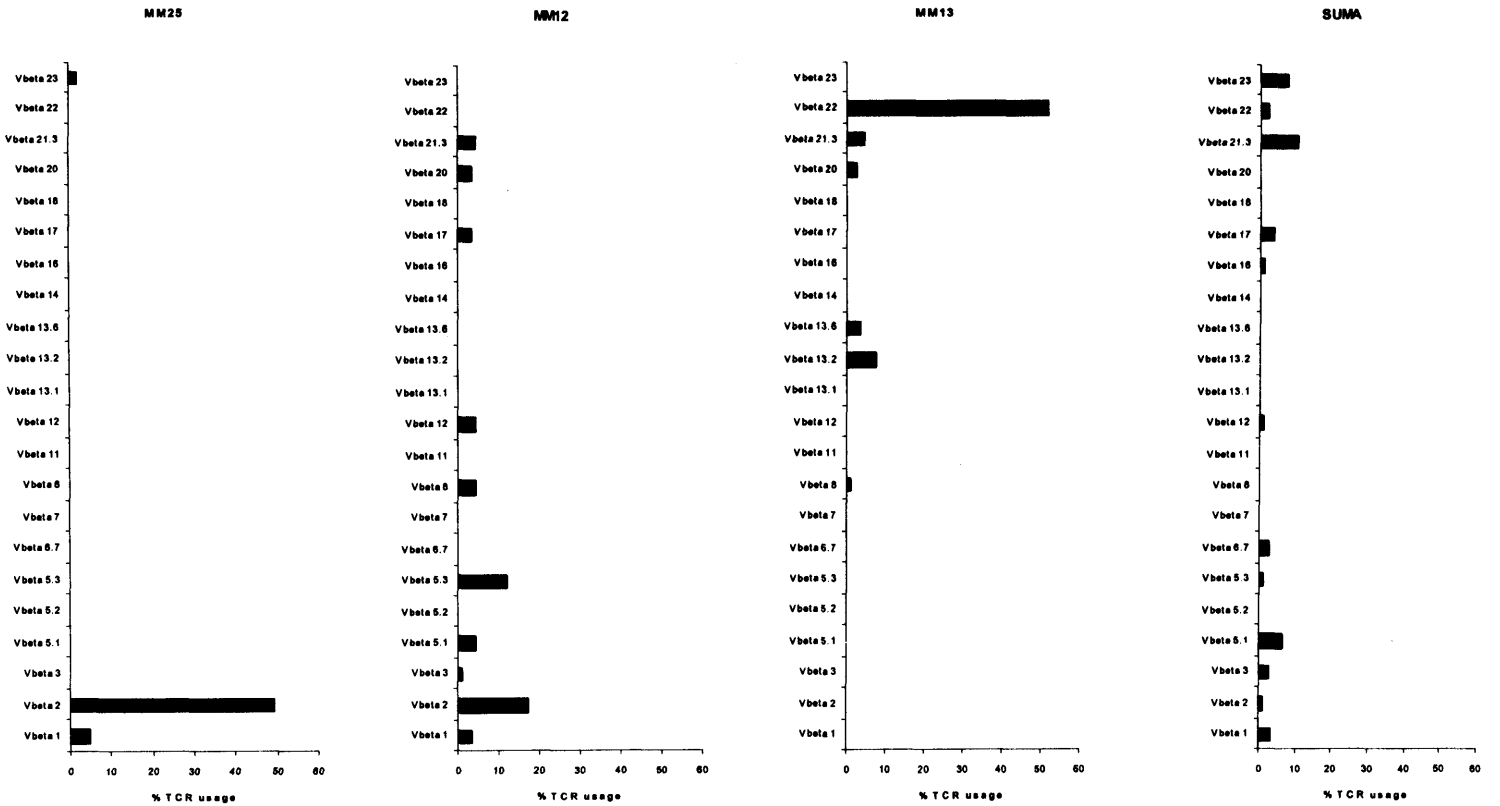


Figure 3.26. Clonal breadth of individual HIV epitope-specific responses in patients who established different persisting viral loads. The Vβ families used by the T cell receptors of cells responding to one HIV epitope at or near the peak of the epitope-specific response were determined for four patients: the A3 RLRPGGKKK response at 31 DFOSx for MM25, a patient who established a high persisting viral load; the A3 QIYAGIKVK response at 40 DFOSx for MM12, a patient who established an intermediate-high persisting viral load; the B8 FLKEKGGL response at 16 DFOSx for MM13, a patient who established a low-intermediate persisting viral load and the B15 MTKGLGISY response at 8-15 DFOSx for SUMA, a patient who established a low persisting viral load. Cryopreserved PBMC were co-stained with an MHC class I tetramer corresponding to the epitope being studied within the patient's early HIV-specific CD8 response, antibodies against CD3 and CD8 and an antibody specific to one of 22 different TCR Vβ families (or an isotype control antibody). The percentage of tetramer positive cells using each Vβ family was determined by flow cytometry, and is shown in the bar charts above, with frequencies <1% regarded as background staining and therefore not shown. The percentage of the entire tetramer positive population detected by staining using the available Vβ family antibodies was 56% (A3 RLRPGGKKK), 57% (A3 QIYAGIKVK), 74% (B8 FLKEKGGL) and 43% (B15 MTYKGLGISY).

strong biasing of the response towards one family, with almost 60% of tetramer positive cells using Vβ 22. The broadest and most evenly distributed Vβ usage was seen with the B15 MTKGLGISY response in patient SUMA, with 12 out of 22 families used, and none of the families accounting for greater than 10% of the total tetramer positive population. It should be taken into consideration however that the percentage of all tetramer positive cells detected by the available Vβ family-specific antibodies only came to between 43 and 74% of the total number of cells responding to each epitope. The breadth of Vβ family usage by the remaining cells is not known.

To gain further insight into TCR usage by HIV-specific T cells in patients who established different persisting viral loads, this analysis was extended to look at responses to more than one epitope per patient. PBMC were stained with other MHC class I tetramers (again at a timepoint near the peak of the epitope-specific response), and in each case, the minimum number of Vβ families involved in the response was determined. Given that in no case were 100% of tetramer positive cells detected by the available Vβ family-specific antibodies, the minimum number of families was taken to be the number detected plus one, as there must be at least one more involved in each case. The results obtained are shown in Figure 3.27 and indicate that there was not a marked difference in the mean minimum number of Vβ families used in epitope-specific responses in different patients. Although the broadest usage of Vβ families was observed in the patient who established a low persisting viral load, responses made by patients in each viral load group exhibited restricted TCR usage, and a two-tailed t-test revealed that overall there was no significant difference in the breadth of TCR usage in the patient who established a low persisting viral load compared to patients who established low-mid, mid-high plus high persisting viral loads (observations from three patient groups combined) ($p=0.073$). Had patient/tetramer availability permitted, this analysis would ideally have been extended to larger numbers of epitopes in more patients, so that more reliable conclusions could be drawn from the results.

3.8 Discussion

The prognostic importance of the persisting viral load established within the first ~six months following infection with HIV-1 (Mellors *et al.*, 1996) indicates that events taking place during the initial stages of this infection are critical determinants of the subsequent disease course. Nonetheless, this phase of

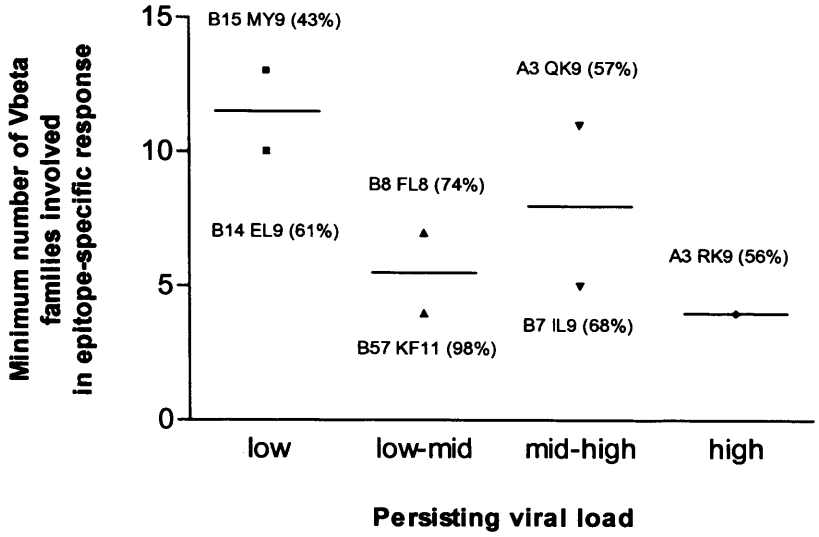


Figure 3.27. Comparison of the breadth of Vβ family usage by the T cell receptors of HIV-specific CD8⁺ T cells in patients who established different persisting viral loads. Vβ family usage by T cells responding to different HIV epitopes was analysed as described in the legend to Figure 3.26 in patients who established differing persisting viral loads. All responses were identified as being part of the patient's early HIV-specific CD8⁺ T cell response as described in the text, and clonal breadth analysis was carried out at a timepoint at or near the peak of the epitope-specific response (determined in chapter 5). The A3 RLRPGGKKK-specific response (A3 RK9) at 31 DFOSx was analysed in MM25, a patient who established a high persisting viral load; the A3 QIYAGIKVK-specific response (A3 QK9) at 40 DFOSx and the B7 IPRRIRQGL-specific response (B7 IL9) at 139 DFOSx in MM12, a patient who established an intermediate-high persisting viral load; the B8 FLKEKGGL-specific response (B8 FL8) at 16 DFOSx and B57 KAFSPEVIPMF-specific response (B57 KF11) at 1125 DFOSx in MM13, a patient who established a low-intermediate persisting viral load; and the B15 MTKGLGISY-specific response (B15 MY9) at 8-15 DFOSx and B14 ERYLKDQQL-specific response (B14 EL9) at 22 DFOSx in SUMA, a patient who established a low persisting viral load. An estimate of the minimum number of Vβ families involved in each individual epitope-specific response was made based on the actual number of Vβ families used by the TCRs, with the addition of one more family for every response where <100% of tetramer-positive cells were identified using the available TCR Vβ family-specific antibodies. Each symbol represents the minimum number of Vβ families used by the TCRs of cells of one epitope specificity. The percentages of the tetramer positive population stained using the Vβ family-specific antibodies available are indicated. Patients are divided into four groups according to the persisting viral load they established at around 180 DFOSx. The mean values for each group are indicated by horizontal bars. Statistical analysis (a two-tailed t-test) revealed that there was no significant difference in the in breadth of Vβ family usage by the T cell receptors of HIV-specific CD8⁺ T cells in the patient with a low persisting viral load compared to patients in other groups.

infection remains poorly characterised and the factor(s) determining the setpoint viral load have yet to be defined.

The HIV-specific $CD8^+$ T cell response is known to play an important role in control of early virus replication (Borrow *et al.*, 1997; Jin *et al.*, 1999; Price *et al.*, 1997; Schmitz *et al.*, 1999). However kinetic, quantitative and qualitative aspects of the primary HIV-specific $CD8^+$ T cell response are all incompletely characterised, and whether/how they may impact on the setpoint viral load remains unclear. The main goal of this study was thus to characterise the primary HIV-specific $CD8^+$ T cell response in a cohort of HIV-1 infected individuals, in order to gain insight into why this response fails to contain viral replication more completely, plus potentially identify aspects of the response that may differ in patients who subsequently establish different persisting viral loads.

The primary HIV-specific $CD8^+$ T cell response was studied using PBMC samples cryopreserved at a series of timepoints from patients recruited following presentation with symptomatic primary HIV-1 infection. All patients were HIV seronegative or indeterminate at the time of presentation. Each sample was assigned a timepoint according to the number of DFOSx at which it was collected, although the viral load and HIV serostatus typically provide a better means of comparing the time during primary infection at which patients were first sampled. In this study, I defined the acute phase of infection as the time during which the acute burst of viral replication occurred, prior to seroconversion; subacute infection as the period when seroconversion was taking place, and the viral load was typically declining; subsequent timepoints when viraemia was reduced and full seroconversion had occurred as early infection. This definition of acute infection is more stringent than that used in many other studies, in which any timepoints during the first few months post-infection have been considered as "acute" (Altfeld *et al.*, 2001a; Goulder *et al.*, 2001a; Papagno *et al.*, 2004).

Sequential PBMC samples were available from acute, subacute or early infection onwards in a total of 16 patients recruited from clinics in London. Of these, two commenced combination ART during primary infection. In addition, limited samples were available from one patient recruited from a US clinic. This patient did not receive any ART and samples were obtained at timepoints from acute infection onwards. rVV-stimulated ELISPOT assays were carried out using samples from sequential timepoints during acute and/or early

infection for ten of these patients. Although these included some individuals who went onto receive ART, only timepoints prior to the commencement of therapy were studied. 13 of the patients were selected for more in-depth studies of the primary HIV-specific $CD8^+$ T cell response. These patients were chosen because they had remained ART-naive throughout the course of infection and because sufficient samples were available for study at timepoints over the course of early (plus subacute and acute where possible) infection. Unfortunately sample availability was more limited in the one patient recruited from the US clinic and only allowed limited analysis to be performed for this patient. Much of the work in this chapter focused on characterisation of the HIV-specific CD8 response during early infection in different infected individuals, with follow-on studies in chapter 5 addressing the kinetics and phenotype of epitope-specific components of the response during acute, subacute and early infection.

The patients were divided into groups according to the viral load established at around six months post-infection. The viral load boundaries on which this categorisation was based corresponded to those defined by Mellors *et al* (Mellors *et al.*, 1996) as the quartile values taken from the frequency distribution of plasma viral load measurements in a cohort of 180 US patients whose plasma viral loads were measured within the first six months of infection. It was notable that the persisting viral loads established in the cohort of London patients I studied were heavily biased towards the upper viral load quartiles from the study by Mellors *et al*, and no patients fell into the lowest quartile (although I was able to carry out limited studies on samples from one patient (in fact recruited from a US clinic) who did establish a persisting viral load in the lowest quartile). It is possible that differences in the locally circulating virus strains and/or ethnicity or socioeconomic circumstances of the US cohort studied by Mellors *et al* and the London patients I studied contributed to the differences in the distribution of persisting viral loads established by ~six months post-infection.

Examination of the composition of the two patient cohorts revealed that both cohorts largely consisted of Caucasian male homosexuals who were predominantly infected with clade B HIV-1 strains. However an important difference between the cohorts that likely made a major contribution to the difference in the persisting viral loads established by the two groups of subjects was in the way they were recruited. The cohort studied by Mellors *et al* was identified by repeated screening of a population of high-risk individuals

for HIV seroconversion, whereas the patients in the London cohort were recruited following clinical presentation with symptoms (e.g. fever, lymphadenopathy, nausea, rash and/or headaches) consistent with acute viral infection following a high risk HIV exposure incident. A highly symptomatic seroconversion illness may well be indicative of a high initial level of viral replication, which may be associated with strong cytokine responses involving for example type I IFNs and IL-1, that are responsible for many of the symptoms characteristic of acute viral infections. If the subjects in this cohort did experience particularly high-level early virus replication, this may have also been associated with the establishment of relatively high persisting viral loads. Indeed, there is evidence in the literature to suggest that individuals experiencing highly symptomatic primary infection do have a poor disease prognosis (Henrard *et al.*, 1995; Pedersen *et al.*, 1989; Vanhems *et al.*, 2000; Vanhems *et al.*, 1998).

Notably, the London patient cohort is biased not only in terms of the distribution of persisting viral loads they established, but also in their HLA types, with HLA alleles that are associated with rapid disease progression, such as HLA-B3502/03 (Gao *et al.*, 2001; Jin *et al.*, 2002) and HLA-B8 (Candore *et al.*, 1998; Kaslow *et al.*, 1990; McNeil *et al.*, 1996) being present at higher than the normal frequency. For example, while the frequency of HLA-B8 in the USA Caucasian population is ~10% (HLA 1991) the frequency of this allele in this patient cohort was 38% (5/13 subjects expressed HLA-B8). This may have contributed to the relatively high viral loads in this patient cohort. It may also influence the repertoire of epitopes that responses were observed against.

Ideally, the 13 patients for which detailed analysis of the primary HIV-specific CD8 response was carried out would have been evenly distributed over the four viral load quartiles defined by Mellors *et al* (Mellors *et al.*, 1996) (which are probably more representative of the spectrum of persisting viral loads established by clade B-infected individuals in the US/Europe than the viral loads established by the London patient cohort). As no patients in the lowest viral load quartile were available for study, it was not possible to address what type of response is associated with good control of early viral replication; the nature of the response and whether/how this may differ in patients could only be studied/compared for patients in the three uppermost viral load quartiles. Limited sample availability meant that in-depth analysis of the one patient

available who established a persisting viral load in the lowest viral load quartile could not be carried out. In addition, one patient in the lowest viral load quartile would not have permitted statistical analysis of any differences observed between that group compared to the other three to be performed, and so I could not hope to make anything other than anecdotal observations.

As reviewed in the introduction to this chapter, a variety of assays are available for analysis of virus-specific CD8 T cell responses. They vary with regard to the antigenic stimulus used, employing entire viral proteins (delivered intracellularly), long (overlapping) peptides corresponding to viral protein sequences, or short peptides comprising individual epitope sequences. The latter only gives information about selected components of the response; the former two approaches are better for obtaining a picture of the overall response. Different assays also vary with respect to the methodology by which antigen-specific responses are detected. Many assays use a functional read-out, but the relatively recent advent of flurorescently-labelled MHC multimers allows for detection of epitope-specific T cells independently of function. In this chapter I used rVV- and overlapping peptide-stimulated IFN-γ ELISPOT assays to obtain an overall picture of the primary HIV-specific $CD8^+$ T cell response in the patient cohort under study.

Initially rVV-stimulated IFN-γ ELISPOT assays were used to assess the HIV-specific $CD8^+$ T cell response. This provided a simple and rapid means of determining responses to five major viral proteins over time in a number of patients. However, the rVV-stimulated IFN-γ ELISPOT assays had a number of drawbacks:

Firstly, the information that can be gained from the results of this somewhat crude assay is limited: because the assay only enables the detection of responses to a limited number of non-autologous viral proteins, an incomplete picture of the total response is obtained; also, because it does not reveal whether the response to a protein is made up of the responses to several epitopes within that protein, it gives no information about the number of epitopes recognised in a patient's response.

Secondly, the assay depends on the processing of HIV proteins delivered into APCs into epitopic peptides that are presented to $CD8^+$ T cells. However, at very early timepoints there may be a limited frequency of APCs in the PBMC population as this is a time where major expansions of HIV-specific $CD8^+$ cells are occurring (Pantaleo *et al.*, 1994). This may account for the weak

responses seen at acute/early timepoints for some patients (e.g. MM19 and MM27) which could be interpreted as an apparent delay in kinetics of expansion of HIV-specific $CD8^+$ responses.

Thirdly, because the rVV-stimulated ELISPOT assay employs a functional read-out, it is only able to detect cells making IFN-γ (this may not be the case for all T cells (Goepfert *et al.*, 2000; Kostense *et al.*, 2001; Shankar *et al.*, 2000)) and perhaps more importantly, only enumerates cells capable of cytokine production on *in vitro* restimulation. In acute HIV infection when cells are highly activated, they may undergo spontaneous apoptosis *in vitro* (Roos *et al.*, 1994), and so it is particularly likely that responses will be underestimated at this time; this again could have contributed to the weak responses observed at early timepoints in some patients.

Fourthly, in experiments carried out to assess the suitability of this method for assessment of T cell responses, it was found that the magnitude of responses detected using this assay were not always very reproducible. This could be due to the two day incubation period with a recombinant vaccinia virus, which may have a negative influence on the *in vitro* survival of the cells in some experiments. In an attempt to address this problem, for each patient, cells from all timepoints of interest were assayed in the same experiment. Nonetheless, all results needed to be interpreted with caution and no conclusions could be drawn about the relative magnitudes of responses in different patients from rVV-stimulated ELISPOT data.

Given the many drawbacks to this assay method, the conclusions that can be drawn from the results I obtained are relatively limited. However, using this technique I did confirm (Borrow *et al.*, 1994; Koup *et al.*, 1994) that HIV-specific CD8 responses could be detected in all patients studied from the earliest timepoint tested (which in some cases was even prior to the peak in acute viral replication). The development of the nAb response during primary HIV infection has also been studied in some of these patients, in which the $CD8^+$ T cell response was detected at least two months earlier than nAb activity (Aasa-Chapman *et al.*, 2005). As CD8 responses begin to be induced as acute-phase viral replication occurs, the nature of this initial response could potentially have a big impact on the efficiency of control of primary viral replication.

In several patients there was evidence that responses may be of relatively low magnitude at the earliest timepoints studied. As discussed earlier, there are a number of potential explanations for this observation (including a paucity of

APCs in the PBMC population and/or the apoptotic tendency of $CD8^+$ T cells at these timepoints). However these observations could potentially reflect relatively slow expansion of HIV-specific CD8 responses in these patients. The kinetics of expansion of epitope-specific T cell responses in primary HIV infection is addressed further in chapter 5.

In addition, the results from these assays highlighted that the virus-specific CD8 response in most patients was not evenly distributed across the five proteins studied, but was frequently concentrated on one or two proteins. There was also some evidence to suggest that the response was more broadly and evenly directed in patients with lower persisting viral loads, although the scope and size of this analysis did not permit definitive conclusions to be drawn about this.

To study HIV-specific $CD8^+$ T cell responses in more detail, ELISPOT assays in which cells were stimulated with overlapping peptides corresponding to the entire HIV-1 clade B consensus sequence were used for further work. This approach shares some of the disadvantages of the rVV-stimulated ELISPOT assay - notably, both are functional assays that only detect cells able to produce IFN-γ and thus do not allow determination of the full magnitude of an epitope-specific T cell response (particularly during acute infection when, as discussed above, many of the responding T cells are highly activated and prone to undergo apoptosis *in vitro*). However it has many advantages compared to the rVV-stimulated ELISPOT assay approach. By screening for responses to peptides spanning the whole HIV genome a more comprehensive approach was taken to study the entire HIV-specific response rather than just focusing upon a limited selection of viral proteins. Another advantage of the use of peptide-stimulated assays is that they do not have the same issues associated with antigen presentation as with rVV-stimulated assays since the HIV antigens do not require such extensive processing and can potentially be presented by any MHC class I-expressing cell type, plus there is no potential competition for presentation by vaccinia viral antigens. Importantly, I also found that the magnitude of responses detected in peptide-stimulated ELISPOT assays was much more reproducible than that in rVV-stimulated ELISPOT assays.

I could have chosen to evaluate peptide-stimulated T cell production of IFN-γ by ICS rather than ELISPOT assay. ICS offers the option of confirming the phenotype ($CD8^+$ versus $CD4^+$) of responding T cells. However, as discussed

below, it was expected that the vast majority of responses detected would be $CD8^+$ T cell-mediated. Further, ELISPOT assays are more straightforward to carry out, and are typically more sensitive than ICS assays.

In this study, peptides based upon the clade B consensus sequence, 20 amino acids in length with an overlap of 10 amino acids, were used. This was thought to be a suitable length and degree of peptide overlap to use following results from a study comparing different sets of overlapping peptides varying in length and overlap for their ability to detect CD8 responses (Draenert *et al.*, 2003). This showed that peptides ranging from 15 to 20 amino acids and in overlap from 10 to 11 amino acids yield similar results (in terms of the breadth and magnitude of responses detected in the entire cohort) in IFN-γ ELISPOT assays (although there may be intra-individual differences in terms of the detection of responses using the different peptide sets). Using 20mer peptides overlapping by 10 amino acids should have enabled detection of responses to all T cell epitopes of 11 amino acids long or less.

The peptides were arranged into pools in matrices for screening in IFN-γ ELISPOT assays. By initially using a peptide pool matrix-based system of screening for patient responses, patients could be rapidly screened for responses to the entire HIV genome using a relatively small number of cells - around $3x10^7$ PBMC (not including cells needed for confirmation of positive responses), the number which might easily be obtained from 50ml blood (compared to 12×10^7 PBMC required if all individual peptides in the panel had been tested individually).

This approach was validated in a study by Addo *et al* (Addo *et al.*, 2003), which showed that no HIV-specific T cell responses were detected in 10 HIV-1 negative subjects when using peptide pools or individuals peptides to stimulate cells; T cell responses to individual peptides detected using peptide matrices correlated well with responses detected by use of individual peptides in 20 subjects tested (verifying that when peptides are included in a pool with other peptides there is no interaction or competition between the peptides which prevents their presentation and ability to elicit a response); and that the responses seen were $CD8^+$ T cell dependent. Due to limited sample availability, these validation experiments were not extensively repeated; however I did check in a small number of patients that the responses to individual peptides were also detected when they were included within a pool of peptides, and that the responses were CD8 T cell-mediated. The issue of

whether responses could be detected to HIV peptides in seronegative individuals is discussed further in chapter 4.

The sequence of the peptides used for the comprehensive epitope mapping corresponded to the HIV-1 clade B consensus sequence, not patient autologous viral sequences. By using non-autologous peptides that did not initially stimulate the responses *in vivo*, there is a risk of biasing the results towards the detection of more conserved epitopes, whilst missing any epitopes in any variable parts of the genome, where the differences between the autologous virus sequence and non-autologous sequence are likely to be the greatest (reducing the likelihood of cross-recognition of the consensus sequence). It is unclear just what proportion of the response was likely to have been missed by the use of non-autologous peptides in ELISPOT assays. This would clearly vary between patients, depending on both the relatedness of their autologous virus sequence to the clade B consensus sequence, and the ability of epitope-specific T cells to cross-recognise non-autologous versions of the epitope peptide. In one patient studied in our laboratory, whose response was originally mapped using peptides corresponding to the autologous acute stage virus sequence, subsequent comparison of the recognition of the autologous sequence, consensus sequence and a laboratory adapted virus sequence of the epitope peptides by patient PBMC showed that by using either of the non-autologous sequences, only 3 out of 39 epitopes would have been missed (Jones *et al*, unpublished data). However, this may have been a reflection of the flexibility of this particular subject's response to recognise potential epitope variants. Other studies have suggested that sequence variability does have an important impact upon the frequency of recognition of responses, with HIV-specific CD8 responses being observed to cluster within more conserved regions of the virus (Yusim *et al.*, 2002). Similarly, comprehensive epitope mapping studies have reported that proteins like Vif, Vpr, Tat, Rev and Vpu were less frequently recognised (Addo *et al.*, 2003; Masemola *et al.*, 2004), possibly reflecting more variability in these genes. By contrast, a study of T cell responses in the SIV macaque model where peptide sequences based on the infecting virus were used found that variable proteins like Tat were frequently targeted (Allen *et al.*, 2000b). Moreover, it has been confirmed that responses to the more variable regulatory and accessory proteins are underestimated when consensus-based peptides, rather than peptides based on autologous virus are used (Altfeld *et al.*, 2003).

In the analysis of results to determine the epitopic regions targeted by the HIV-specific response, it was decided that the positive responses would be defined as those which were greater than three times the background level of IFN-γ production (i.e. that stimulated by medium alone) and greater than 50 SFC per 10^6 PBMC. These are the same criteria as used by most other similar studies (Addo *et al.*, 2003; Altfeld *et al.*, 2003; Edwards *et al.*, 2002), but not all. For example, others have defined positive responses as those greater than twice the response of negative control wells (cells incubated with either no peptide or a pool of irrelevant peptides) (Cao *et al.*, 2003) (although this did not raise the number of responses seen relative to other studies); or those which exceed the mean of negative control wells plus three standard deviations (Frahm *et al.*, 2004). In one study, the background level of response was determined using a panel of HIV seronegative subjects to define the spread of negative responses to each peptide pool, and the mean value plus four standard deviations used as the threshold for positive responses.

A further decision I made was that when establishing the epitopic regions recognised by each subject, the responses seen to two overlapping adjacent peptides would be counted as one response. Because of this, the number of epitopes recognised is likely to have been underestimated (also, more than one epitope may be contained within one epitopic region). However, a more conservative approach was thought to be better than overestimating responses. Such differences in analysis of data and how a positive response is defined may influence the conclusions made from studies carried out by different groups.

The total magnitude of the early response (as detected by IFN-γ ELISPOT assay) in the 13 patients studied was found to range between 1,500 and 21,000 SFC per 10^6 PBMC, with a mean of around 10,800 SFC per 10^6 PBMC. The upper end of this range was similar to that found in a similar study by Addo *et al* (Addo *et al.*, 2003) which comprehensively mapped the epitopic regions recognised by 57 HIV-infected subjects at different stages of infection using IFN-γ ELISPOT assays (although in this study, the strongest response was observed in an untreated patient with chronic non-progressive infection). However the largest response observed another study by Cao *et al* (Cao *et al.*, 2003) (where like my study, responses were evaluated during acute and early infection) was much lower (around 3000 SFC per 10^6 PBMC). 10,800 SFC/10^6 PBMC corresponds to ~1% of PBMC (or 2-5% of peripheral blood

$CD8^+$ T cells). Further, for the many technical reasons discussed above, this is likely to be an underestimate of the true magnitude of the HIV-specific CD8 response. These results thus suggest that HIV-specific CD8 T cell responses are typically of extremely high magnitude during early infection.

No correlation was found between the magnitude of the response and the concurrent viral load in this study, suggesting that the frequency of HIV-specific CD8 cells is not a reflection of antigenic load. This is in agreement the findings of some groups (Addo *et al.*, 2003; Frahm *et al.*, 2004; Gea-Banacloche *et al.*, 2000; Novitsky *et al.*, 2003), but not others, who have found there to be a positive (Betts *et al.*, 2001; Buseyne *et al.*, 2002; Masemola *et al.*, 2004) or inverse correlation (Edwards *et al.*, 2002; Greenough *et al.*, 1997; Ogg *et al.*, 1998) between the frequency of HIV-specific responses and viral load in chronically infected individuals.

From the inter-group analysis, there was a suggestion that there may be a relationship between the magnitude of the early HIV-specific CD8 response and the persisting viral load established by ~six months post-infection, although a significant difference was only seen when comparing the intermediate to high with the high viral load patients but not the low to intermediate and high viral load patients, giving some ambiguity over the results. Overall, there was no evidence for the magnitude of the virus-specific CD8 T cell response in early infection being a key determinant of the efficiency of viral control.

It would have been interesting to have been able to measure the magnitude of the response during acute and/or subacute infection to see whether the size of the response at this stage is more critical. It should also be taken into consideration that it is possible that responses may not be expanded with the same kinetics in different patients. The kinetics of expansion of epitope-specific components of the primary HIV-specific CD8 response is explored in chapter 5.

Looking at the breadth of the responses detected, the number of epitopic regions found to be recognised by the patients in this study ranged between 3 and 17. This was much lower than the largest number of responses detected in the study by Addo *et al* (42) (Addo *et al.*, 2003). Although the maximum that could have been detected in this study was 30 due to the limit put on the number of peptides re-tested following the initial testing of peptide pools, none of the subjects had responses reaching this number. That my study did not detect as many responses as others is also evidenced by the finding that only

20% of the genome was found to be immunogenic as compared to 63% peptides being targeted in the study of Addo *et al.* Similarly, the breadth of the response in terms of the number of protein subunits recognised was also found to be lower here (up to 8 subunits out of 14 recognised) than in the Addo study (13). However, it is notable that the study of Addo *et al* included chronically-infected individuals in addition to acutely-infected individuals, and the broadest responses were observed in the context of untreated chronic infection. Likewise other studies have indicated that the epitope breadth of the CD8 T cell response in primary HIV infection may be more limited than that in chronic infection (Altfeld *et al.*, 2001a; Dalod *et al.*, 1999b), e.g. a mean of 2.3 epitopes (range 0-6) were recognised by patients in primary infection in the study of Cao *et al* (Cao *et al.*, 2003). A narrowly directed response was also found in the context of primary infection in the study of Yu *et al* (Yu *et al.*, 2002), where virus specific T cell responses in one patient were found to be narrowly directed against two epitopic regions, illustrating that initial high-level viraemia does not necessarily induce broadly directed responses. Responses may be typically narrower in the primary compared to during the chronic stage of infection. Defects in antigen presentation/CD4 help in primary infection may limit the number of responses that can expand during this stage of infection (this idea is explored in chapter 4). The broadening of the response over time may parallel increases in viral diversity as a result of the error-prone RT enzyme. Broadening of the T cell repertoire may also reflect responses of the immune system to viral escape from responses that are immunodominant during acute and early infection.

No correlation was observed for the 13 patients studied here between the breadth of the HIV-specific CD8 response in early infection and the viral load (either concurrent or persisting). Other studies (some in chronically-infected individuals) have also found no correlation between the breadth of the response and the concurrent viral load (Addo *et al.*, 2003; Cao *et al.*, 2003; Dalod *et al.*, 1999a; Frahm *et al.*, 2004; Masemola *et al.*, 2004). However, this is again a subject of debate since others report that the breadth of the total response is inversely associated with the concurrent viral load (Edwards *et al.*, 2002; Altfeld *et al.*, 2001a). The breadth of the response in *acute* infection (which is influenced by the kinetics of expansion of different epitope-specific responses) may be much more critical than the breadth in *early* infection.

This study did however find there to be significant positive correlation between the breadth and magnitude of the response in accordance with the findings of others (Cao *et al.*, 2003; Addo *et al.*, 2003). The latter study found that a broader and stronger response was observed in the context of continuous exposure to antigen, with the strongest and broadest responses observed in those with untreated chronic infection, and lower and more narrowly directed responses detected in those treated during primary infection.

As this discussion has highlighted, there is much conflict in the literature about the relationship between HIV-specific $CD8^+$ T cell responses with plasma viraemia. Such disagreement may be attributed to differences in the way the studies were performed. Whilst more recent studies have used peptides corresponding to the consensus sequence of the entire HIV genome, many earlier studies have used single peptides, restricted panels of optimal epitopes or a limited selection of proteins to assess responses. The use of different techniques for assessing responses may also have an impact, for example, ICS may utilise co-stimulatory molecules that are not used in ELISPOT assays and this may mask *in vivo* immune response differences that may exist among subjects. A major reason for the discrepancies is also likely to be the use of different study populations. Some studies but not others utilise patients on treatment; there may be differences in the frequencies of HLA alleles within populations (leading to potential differences in immune responses among subjects with different HLA types); and the subjects involved may be at different stages of infection.

The findings discussed above suggest that neither the magnitude nor epitope breadth of the HIV-specific $CD8^+$ T cell response in early infection correlate with the degree of virus containment, and so it is possible that the magnitude, breadth and/or kinetics of the response in acute infection (or perhaps qualitative aspects of the response) may be of more importance.

Out of 14 HIV protein subunits tested, all but Protease and Vpu were found to be recognised by at least one subject in the study population. Another study also did not detect any Vpu-specific responses in a cohort of acutely-infected patients (Cao *et al.*, 2003), although others have observed that all proteins serve as targets for the HIV-specific response (Addo *et al.*, 2003; Betts *et al.*, 2001; Masemola *et al.*, 2004). The general consensus among most studies is that Nef is the most frequently recognised subunit, with responses to Vpu being the least frequently detected, so the lack of Vpu-specific responses

seen in this study and that by Cao *et al* may reflect the relatively low frequency of responses to Vpu, and the low number of subjects in which responses were studied.

The area of the genome found in this study to be most frequently targeted was in Nef: YKAAVDLSHFLKEKGGLEGL. Similarly, the most immunodominant peptide in the study by Addo *et al* (Addo *et al.*, 2003) was TYKAAVDLSHFLKEK. In terms of the total magnitudes of responses induced to each protein subunit among the entire study population here, Nef was also found to induce the highest cumulative magnitude response, highlighting the dominance of Nef-specific responses within this cohort. That Nef was found to be a dominant target for CD8 responses was perhaps not surprising, since many epitopes have been defined in this protein, and it has a high density of epitopes per peptide length (HIV Molecular Immunology Database, 2003). That Rev induced the second highest cumulative magnitude response overall argues against the idea that the relative levels of sequence conservation in different parts of the genome dictate the prominence with which they are observed to be recognised in infected individuals.

The preferential targeting of Gag was found to be significantly associated with viral control in one study of a cohort of chronically infected patients (Masemola *et al.*, 2004). Others have also reported an association between higher magnitude (and broader) anti-Gag p24-specific T cell responses and lower plasma viral loads during chronic infection (Edwards *et al.*, 2002; Novitsky *et al.*, 2003). This however was not found to be true for this study population when then the proportion of the early response targeting Gag (or Env, Pol or Nef) in the 13 patients was plotted against their persisting viral load.

One explanation for the reported association between Gag-specific responses and better viral control may be that Gag p24 has functional constraints which mean it does not readily accommodate new mutations and remains relatively conserved, making it difficult for the virus to escape from Gag-specific responses (Goulder & Watkins, 2004). This would allow for control of virus by the induction of a Gag p24-specific response.

Some studies have also reported an association between Nef-specific responses and poorer viral control, with preferential targeting of Nef in those with higher viral loads (Masemola *et al.*, 2004) or higher Nef-specific T cell responses being associated with a higher viral load (Novitsky *et al.*, 2003). Even though the proportion of the Nef-specific response was not found to be correlated with the persisting viral load in my study, the domineering nature of

anti-Nef responses in terms of frequency of recognition and cumulative responses at early stages of infection may be noteworthy in this context. Nef is quite a variable protein, so it could be speculated that viral escape from immune recognition may more easily occur in epitopes within Nef. If early immune responses targeting Nef do not serve to efficiently control replication due to the more rapid viral escape from these responses, this in turn may impact upon long term viral control.

Comparisons of the protein/epitope specificity of HIV-specific CD8 responses in chronic compared to primary infection have been made. Differences have been observed both in the pattern of protein recognition (initial preferential targeting of Nef during primary infection, followed by an increased contribution of other proteins in the chronic phase, with Gag p24-specific responses becoming dominant (Addo *et al.*, 2003)) and in the specific epitopes recognised (Goulder *et al.*, 2001a). These finding are consistent with there being early escape from initially dominant (e.g. Nef-specific) responses, followed by expansion of responses of other specificities (including certain Gag epitopes).

$CD8^+$ T cell responses which target epitopes within proteins that are expressed early during the viral lifecycle (such as Nef, Tat and Rev) have been reported to control viral replication *in vitro* more effectively than responses which target epitopes within proteins which are expressed later in the lifecycle (van Baalen *et al.*, 2002). The *in vivo* importance of this may be that $CD8^+$ T cells specific for early proteins may have a longer window of opportunity to recognise and kill the infected cell before new viral progeny are made and released, and also that epitopes may be presented before immune evasion mechanisms are active, e.g. downregulation of MHC class I molecules. Indeed, there is also evidence to suggest that $CD8^+$ T cell responses directed at epitopes in early viral proteins may be particularly efficacious *in vivo* too, with observations of slower disease progression in HIV-1 infected patients who exhibited strong CTL responses to early proteins (van Baalen *et al.*, 1997).

In the light of this, the relationship between responses targeting early proteins and the efficiency of early viral control was investigated. When the proportion of the early response targeting epitopic regions within Nef, Tat and Rev was calculated for each patient, the results were quite variable within the subjects in the same viral load group; and the mean percentage of the response

targeting early proteins was not significantly different between the three patient groups. The low frequency of recognition of epitopes within Tat in Figure 3.21 may suggest that potential responses to the variable Tat protein could be overlooked by using peptides based on the consensus sequence for assessment of T cell responses; in turn this could influence the observations made on responses to early expressed proteins.

The analysis of the breadth of the response using rVV-stimulated ELISPOT assays suggested that there was biasing of the response towards epitopes in one or two immunodominant proteins. That the primary HIV-specific response tends to be focused on a few immunodominant regions of the HIV genome was confirmed in the peptide-stimulated ELISPOT assays, firstly at the protein level and then the epitope level. A high proportion of the response (>50%) was found to be focused upon a small number or proteins (2) or epitopes (4) in all patients.

Another measure of the breadth of the HIV-specific response, the repertoire of T cell clones used in epitope-specific responses, was also studied, by assessing the usage of different Vβ families by the TCRs of tetramer-stained cells using Vβ family-specific antibodies. This analysis revealed that there was no association between the number of Vβ families used in HIV epitope-specific responses and the persisting viral load established by the patient. However, the conclusions which can be drawn from these results are limited, for a number of reasons. First, not all T cells involved in each response could be detected using this technique, due to the lack of antibodies specific to certain Vβ families. The proportion of the response that was unaccounted for varied for different responses studied, from 2% to 57%; Vβ family usage by remaining T cells was unknown, and could have had a significant impact on the results. Second, this technique does not reveal the total number of T cell clones participating in a response, since more than one clone of cells may be involved in the response in a given Vβ family. The full extent of the use of different T cell clones could have been assessed by more complex techniques, such as TCR heteroduplex analysis or TCR sequencing of sorted tetramer-positive cells (Kalams *et al.*, 1994; Lopes *et al.*, 2003; Turner *et al.*, 2005). Also, the number of patients and epitopes studied here was very limited, so this type of analysis would need to be extended to more patients and epitopes to enable meaningful conclusions to be drawn from the results.

Prior studies in the literature provide support for a relationship between the breadth of T cell clones involved in the HIV-specific CD8 T cell response and the efficiency of control of virus replication. Pantaleo *et al* observed monoclonal/oligoclonal expansions of TCR Vβ families in primary infection in patients who went on to establish high but not low persisting viral loads (Pantaleo *et al.*, 1994; Pantaleo *et al.*, 1997b). This suggests that the primary T cell response in patients who controlled viral replication well involved more synchronous expansion of multiple T cell clones, i.e. was more polyclonal and/or directed against more viral epitopes. Further support for more polyclonal responses being associated with good control of viral replication comes from Lopes *et al* (Lopes *et al.*, 2003), who showed that epitope-specific CD8 T cell responses in HIV-2 infected patients (who typically control viral replication well) were typically more polyclonal than those in HIV-1 infected patients. Although it is not clear whether these associations are a consequence and/or a cause of the good control of viral replication, polyclonal responses could contribute to efficient containment of viral replication by giving potential for recognition of multiple variants of the epitope peptide and thus helping to restrict escape (although there are examples of responses of restricted clonality that are very functionally flexible (Dong *et al.*, 2004)).

One reason why no associations between the persisting viral load and any single parameter studied were seen could potentially be because there was not enough of a spectrum of viral loads represented in the patient cohort. Had there been a number of patients available in the lowest viral load quartile defined by Mellors *et al* (Mellors *et al.*, 1996), then perhaps more differences would have been apparent. It may be that the majority of patients make a similar type of response, and it is only the ones who control early viral replication particularly well (perhaps only the 10-20% of infected individuals who go on to become LNTPs) who have a qualitatively or quantitatively different primary HIV-specific CD8 response. Previous observations made in one patient (SUMA) who did establish a persisting viral load in the lowest quartile lend some support to this hypothesis. In patient SUMA a broad, non-biased response was seen in the rVV-stimulated ELISPOT assay (Figure 3.7). This was reflected by the finding, when his response was more finely mapped, of a response that was broad and co-dominant, targeting over 40 epitopes ((Jones *et al.*, 2004) and unpublished data of Jones *et al*). However these results were obtained from experiments using reagents corresponding to the

patient's autologous viral sequence, and so cannot be directly compared to the results obtained here.

The results from this study were obtained using cells cryopreserved during early infection. As indicated earlier in the discussion, one question of critical importance is how the HIV-specific $CD8^+$ response might evolve over the course of primary infection. In a study by Cao *et al* (Cao *et al.*, 2003), cross sectional analysis of patients showed that the number of epitopes recognised in primary infection was small, but increased with the duration of infection. Another study reporting a kinetic analysis of responses to epitopes in three viral proteins in three infected individuals again suggested that the HIV-specific CD8 response was highly biased towards a small number of immunodominant epitopes in acute infection, and increased in breadth/co-dominance over time (Jones *et al.*, 2004). It is thus possible there are important differences in CD8 responses in acute infection between patients that are no longer apparent by early infection. There have not been any studies that have addressed the evolution of the entire response from acute to subacute, through to early infection in many different patients, so the range of variation in magnitude and epitope breadth of the response and kinetics of evolution of these parameters and whether/how they may relate to the persisting viral load subsequently established is not known.

In summary, characterisation of the nature of the early HIV-specific $CD8^+$ T cell response in patients who establish low-intermediate up to high persisting viral loads using IFN-γ ELISPOT assays indicated that a high magnitude response is typically present in this phase of infection, but revealed little difference in the magnitude and/or breadth of response among the patients available for study here. Although this does not support the idea that the nature of the early response is related to the efficiency of viral control, had analysis of the response at the peak of infection and/or using patients with very low persisting viral loads been feasible, this may have revealed otherwise. Questions about other aspects of the response remain, such as how the kinetics of expansion of different responses in different patients compare, and whether other qualitative aspects of the response differ. Some of these are addressed in chapter 5.

The analysis in this chapter also revealed that the majority of patients make a virus-specific CD8 response that is focused upon a limited number of viral proteins/epitopes in early infection. The reasons for this, and the possible

consequences of making a restricted response, are the subject of experiments and discussion in the next chapter.

Chapter 4

Investigation of mechanisms that may account for the biasing of the primary virus-specific $CD8^+$ T cell response in the majority of HIV-1 infected patients towards a small number of immunodominant epitopes

4.1 Introduction

As reviewed in the main introduction to this thesis and discussed in chapter 3, current data give a very incomplete picture of the expansion of the HIV-specific $CD8^+$ T cell response in primary infection. A number of the studies have shown that the response starts to be induced in the acute phase of infection (Borrow *et al.*, 1994; Koup *et al.*, 1994), an observation that I also confirmed in chapter 3. However, the number of viral epitopes to which responses are induced in acute/subacute infection and the relative kinetics with which different epitope-specific responses expand, plus how these parameters may vary in different individuals are relatively poorly understood. Pantaleo *et al* (Pantaleo *et al.*, 1994; Pantaleo *et al.*, 1997b) observed monoclonal/oligoclonal TCR Vβ family expansions during acute/subacute infection in patients who subsequently controlled viral replication poorly, but not those who went on to establish low persisting viral loads, suggesting that responses of limited clonality to a very small number of viral epitopes were rapidly expanded during primary infection in the former individuals, whereas the response mounted in the latter patients was broader (at the clonal and likely also the epitope level) from the start. In line with this idea, in-depth studies carried out in three infected individuals (Borrow *et al.*, 1997; Jones *et al.*, 2004) indicated that in acute/subacute infection the virus-specific $CD8^+$ T cell response in the two patients who went on to establish high persisting viral loads was highly focused on a limited number of viral epitopes, with delayed expansion of responses of additional specificities, whilst responses to a broader range of viral epitopes were apparent from acute infection onwards in the patient who controlled early viraemia more efficiently. These limited studies (which are extended in chapter 5) suggest that in many HIV-infected individuals there may be preferential expansion of responses to a small number of immunodominant viral epitopes in acute/subacute infection, with delayed expansion of responses of additional specificities over time. The observations I made in chapter 3, where I found that in a panel of infected individuals, all of whom established moderate or high persisting viral loads, a mean of only 8 epitopic regions were recognised (range 3-17) in early

infection, and a high proportion of the response (>50%) was focused on 1-4 immunodominant epitopes; plus results from other studies that have indicated that the epitope breadth of the virus-specific $CD8^+$ T cell response in primary HIV infection is more restricted than that in chronically-infected individuals (Altfeld *et al.*, 2001a; Cao *et al.*, 2003; Dalod *et al.*, 1999b), would fit with this hypothesis.

Building upon these observations, in this chapter I went on to explore mechanisms that may account for there being preferential expansion of $CD8^+$ T cell responses to a limited number of immunodominant viral epitopes during primary HIV infection, and why a broader response may be observed in patients controlling the acute viral burst more efficiently.

A number of factors influence the epitopes against which T cell responses are generated and the immunodominance of individual epitopes within the $CD8^+$ T cell response made to a virus (reviewed by (Chen *et al.*, 2000)). These include (i) factors that influence the array of epitope peptides bound to MHC class I molecules on the surface of the APCs involved in priming the $CD8^+$ T cell response; (ii) factors affecting the interaction between epitope-specific T cells and APCs and (iii) factors that dictate the pre-existing T cell repertoire (including the number and properties of epitope-specific T cells).

The peptides which have the potential to act as T cell epitopes and the abundance with which each is generated and presented with MHC class I on the APC surface will be dictated by the host MHC haplotype, the abundance with which viral proteins are produced and their availability for processing/presentation, the efficiency with which particular peptides are generated by antigen processing pathways, and competition between viral (and host) peptides for binding to MHC (in which the affinity of epitope binding to MHC plays an important role).

In addition, the number and T cell stimulatory capacity of APCs are also important determinants of epitope presentation. Properties of APCs which determine how immunostimulatory they are include the amount of MHC class I and co-stimulatory molecules they express, plus their ability to produce soluble factors.

Finally, features of the pre-existing host T cell repertoire can also influence the immunodominance of responses. The generation an epitope-specific response depends on the existence of epitope-specific T cells within the repertoire. Their frequency, affinity for peptide-MHC complexes and ease of activation

may influence which T cell clones can respond strongly/more rapidly. The presence of competing T cells specific for other epitopes may also be of importance, since they could suppress the response by physically excluding other T cells from contacting their antigen or co-stimulatory ligands, or competing for soluble factors produced by APCs. Competing T cells could even actively suppress the response of other T cells by secreting immunomodulatory cytokines or by destroying APCs, thereby preventing competitor T cell activation.

In primary HIV infection APC numbers and/or function may be compromised (Pacanowski *et al.*, 2001). This could be due to direct or indirect effects of the virus on APCs and/or due to impairment of HIV-specific CD4 responses, which may limit the help available for APC activation.
So, under these conditions, is the competition between T cells for APCs more fierce and is there more biasing of responses towards epitopes which are able to be presented particularly well and/or are recognised by T cell clones that can outgrow their competitors? In patients who are more efficient in controlling early viral replication, APC function may not be as adversely affected. This may mean that there is less competition between T cells of different specificities, and consequently more opportunity for the expansion of a broader response. This may explain the differences in the breadth of the response expanded in patients who control viral replication with differing efficiency (Jones *et al.*, 2004; Pantaleo *et al.*, 1997b).

In the setting of limited availability of activated APCs and a deficit in T cell help envisaged to exist in many patients during primary HIV-1 infection, there may be particularly prominent expansion of responses to peptides which bind with high affinity to MHC molecules and/or which are recognised by high affinity T cell receptors, as these factors may potentially favour T cell activation under suboptimal conditions. In support of this idea, there is evidence that $CD8^+$ T cell responses to high affinity epitopes are less dependent on help for their expansion than responses to lower affinity epitopes (Franco *et al.*, 2000).
In addition, there may be prominent expansion of responses to epitopes that can be cross-recognised by pre-existing memory T cells. There is evidence in the literature for cross-recognition of epitopes in different viruses by $CD8^+$ T cells (Selin *et al.*, 1994; Selin *et al.*, 1998; Wedemeyer *et al.*, 2001; Yang *et al.*, 1989), and in mouse models this has been shown to affect the relative

dominance of epitope-specific responses to subsequent virus infections (Brehm *et al.*, 2002) (discussed in more detail later). It might be envisaged that cross-reactive memory T cells, being present at higher frequencies and being in a more highly activated state than naïve T cells, would have a particularly marked expansion advantage under conditions where APC functions are limiting.

In this chapter, the relationship between the immunodominance and functional avidity of HIV-specific T cell responses was explored, and whether there was greater biasing towards high avidity responses in patients with the highest viral loads (those in which APC stimulatory capacity is likely to be affected the most) was investigated. Mouse models were also used to investigate whether the response to virus infection was biased to high affinity epitopes when help for APC activation was limited. To explore the possibility of cross-reactive immunity being one of the mechanisms contributing to the extreme immunodominance of responses to certain HIV epitopes in acute/subacute infection, responses that were particularly immunodominant in primary HIV infection were studied to see if they may represent expansion of cross-reactive memory responses. Mouse models were also used to look at whether cross-reactive memory responses are preferentially expanded in response to a secondary virus infection if the response is induced when help is limiting.

4.2 Investigation of the relationship between the immunodominance and functional avidity of CD8$^+$ T cell responses

As explained in section 4.1, it was hypothesised that the relative immunodominance of epitope-specific responses within the primary HIV-specific CD8$^+$ T cell response may be influenced by the affinity of the epitope for the presenting HLA allele and/or the affinity of the receptors of responding T cells for the peptide-MHC complex. To test this hypothesis, it was decided to compare the epitope and/or TCR affinity of responses identified in chapter 3 as being dominant and subdominant in early HIV infection in different patients. Ideally, it would have been preferable to perform a comparison of the epitope-specific responses that expanded first in acute infection and later-expanding responses, but as data on the relative kinetics of expansion of epitope-specific responses within each patient was not available, it was assumed that the relative immunodominance of responses in early infection would provide a

reasonable view of their "competitiveness" in the primary HIV-specific $CD8^+$ T cell response.

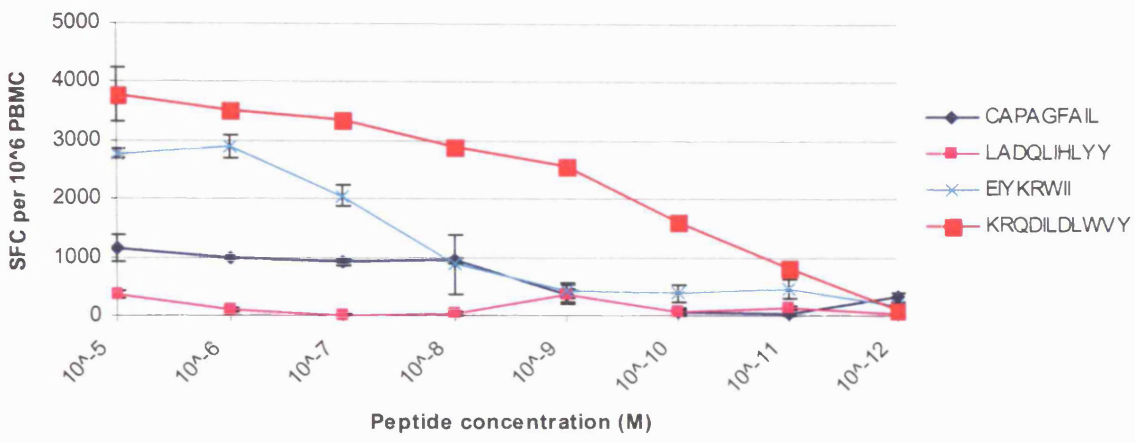
There are a number of assays that can be used to measure the binding affinity of an epitope peptide to its MHC restriction element, including competitive binding assays (Gairin *et al.*, 1995; van der Most *et al.*, 1998) and assays based on stabilisation of empty MHC molecules on the surface of a TAP-deficient cell line (Gairin *et al.*, 1995; Hudrisier *et al.*, 1996). The affinity of TCR binding to peptide-MHC can also be explored, e.g. by measuring the dissociation of bound tetrameric peptide-MHC complexes (Kerry *et al.*, 2003; Reichstetter *et al.*, 2000; Savage *et al.*, 1999), or using surface plasmon resonance in BIAcore analysis (Kerry *et al.*, 2003; Khilko *et al.*, 1993; Tissot *et al.*, 2000). However, because there were many different HIV-specific responses made by this cohort of patients, involving a diverse range of HLA alleles, measurement of the affinity of MHC binding of each epitope and affinity of the responding TCR(s) for peptide-MHC was not feasible. Instead, it was decided that the relative functional avidity of the T cell response to each epitope (which could be gauged by determining the concentration of peptide required to stimulate a half-maximal response by epitope-specific T cells) would be studied, and the relationship between the functional avidity of responses and their relative immundominance within the whole HIV-specific $CD8^+$ T cell response explored. The functional avidity of an epitope-specific $CD8^+$ T cell response is influenced by both the affinity of the peptide for its restricting MHC molecule and the avidity of the interaction between the responding T cells and stimulating cells.

In Figures 4.1– 4.3 (uppermost panels) the results of experiments measuring the functional avidity of the T cell response to different epitopes shown in chapter 3 to be recognised in early HIV infection in patients who established high (Figure 4.1), intermediate-high (Figure 4.2) and low-intermediate (Figure 4.3) persisting viral loads are shown. In each patient only those responses for which the optimal epitope sequence had been determined (Figures 3.11-3.13) were studied, and the cells used were from a timepoint in early infection (in the majority of cases between 3 and 9 months FOSx), so as to minimise the skewing of results due to changes in the affinity of TCRs of T cells involved in epitope-specific responses over time.

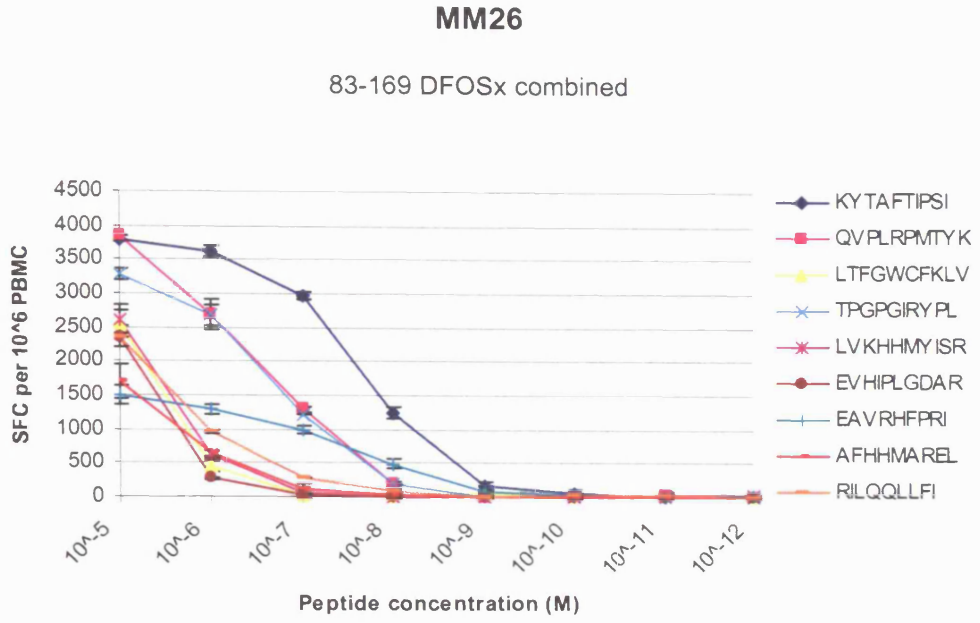
In the lowermost panels of each figure, the functional avidities of each epitope-specific response (calculated from the results of the peptide titrations) are summarised, and the relative immunodominance of the response within the

MM24

125 DFOSx



Epitope peptide	Relative immunodominance of epitope-specific response in early infection (%)	Concentration of peptide required to stimulate half maximum response
CAPAGFAIL	30.8	10^{-9}M
EIYKRWII	26.3	10^{-8}M
KRQDILDLWVY	13.3	10^{-10}M

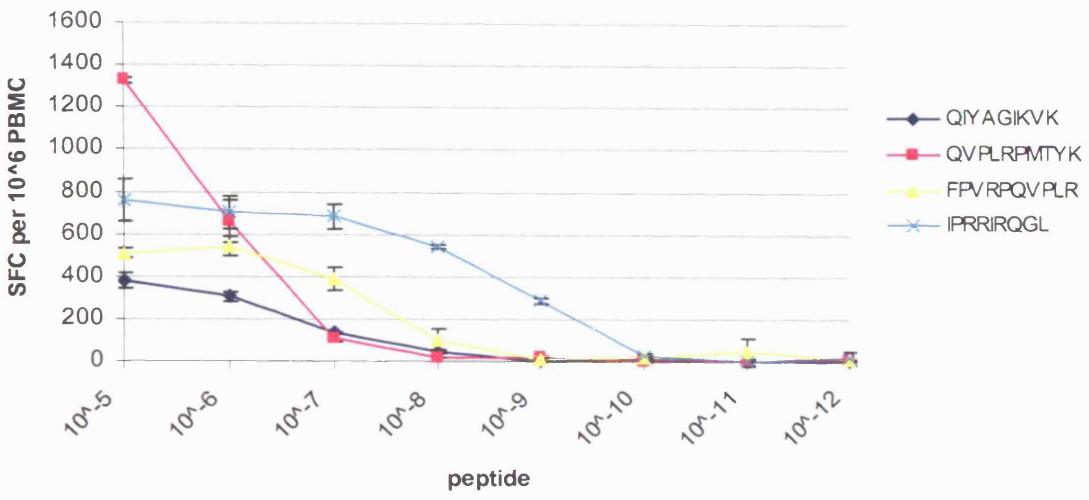


Epitope peptide	Relative immunodominance of epitope-specific response in early infection (%)	Concentration of peptide required to stimulate half maximum response
KYTAFTIPSI	13.3	10^{-8}M
QVPLRPMTYK	16.2	10^{-7}M
LTFGWCFKLV	22.5	10^{-6}M
TPGPGIRYPL	22.5	10^{-7}M
LVKHHMYISR	7.0	10^{-6}M
EVHIPLGDAR	5.9	10^{-6}M
EAVRHFPRI	5.3	10^{-7}M
AFHHMAREL	3.3	10^{-6}M
RILQQLLFI	3.3	10^{-6}M

Figure 4.1. Relative immunodominance and avidity of T cell responses made to HIV epitope peptides by patients with high persisting viral loads. In chapter 3, HIV-1 peptides against which $CD8^+$ T cell responses could be detected during early infection in patients MM24 and MM26 were identified, and the relative immunodominance of the response to each peptide was determined by IFN-γ ELISPOT assay (data in Figures 3.11(a) (MM24) and 3.11(e) (MM26)). The optimal epitopes recognised within some of the peptides against which responses were directed were also identified. In the uppermost panel of each page, the relative avidity of the T cell response to each of these epitopes was determined, by stimulating patient PBMC (cryopreserved at the timepoints indicated) with peptide concentrations between 10^{-5}M and 10^{-12}M, and determining the magnitude of the response elicited by IFN-γ ELISPOT assay. The results shown are expressed as the mean (of duplicate wells) number of specific SFC per 10^6 PBMC (i.e. background values from medium only wells have been subtracted) ±1 SD. The table in the lowermost panel of each page summarises the relative immunodominance of the response to each epitope peptide in early infection (i.e. magnitude of the response to this epitope expressed as a percentage of the total response to all peptides to which responses were detected in this patient) and the relative avidity of each response (concentration of peptide required to stimulate a half-maximal response, calculated from the results of the peptide titrations).

MM12

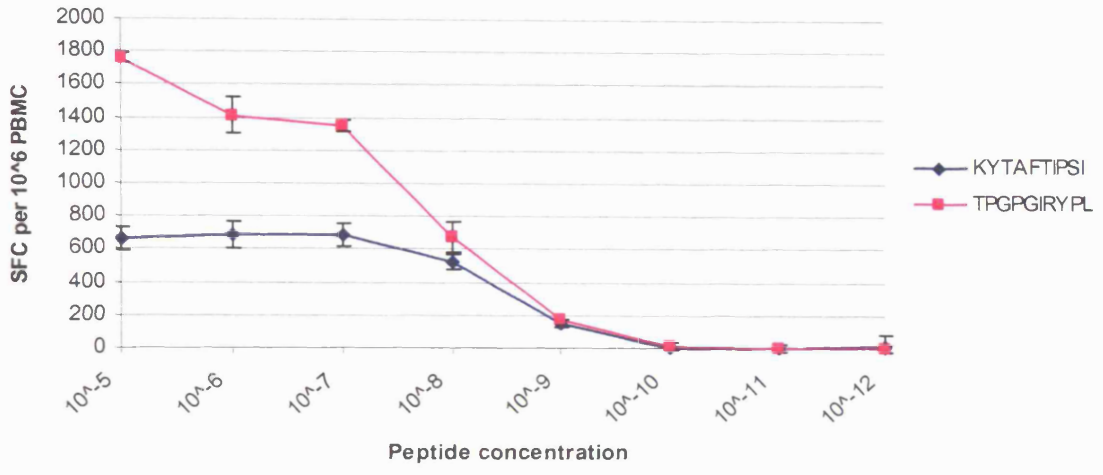
230-321 DFOSx



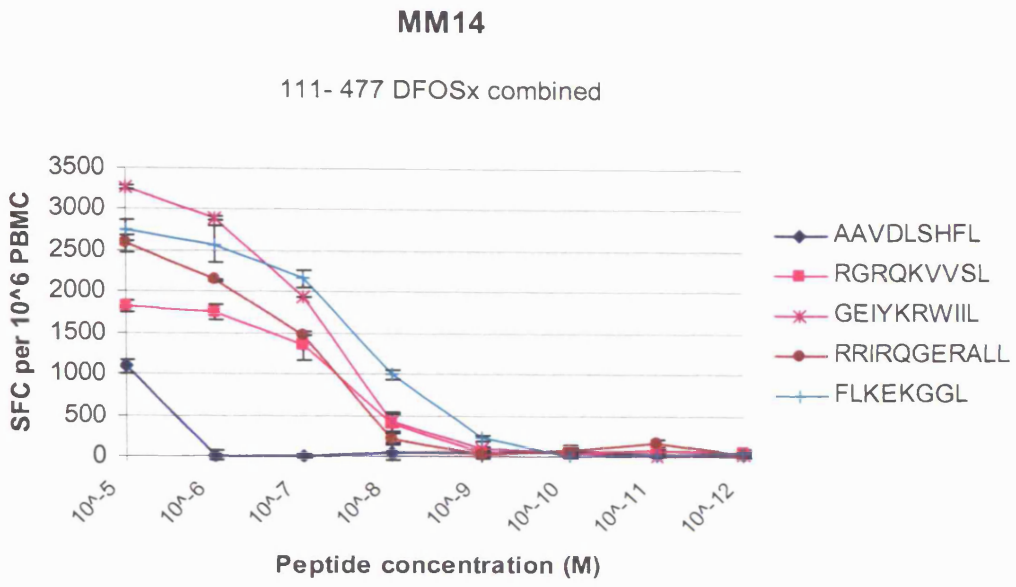
Epitope peptide	Relative immunodominance of epitope-specific response in early infection (%)	Concentration of peptide required to stimulate half maximum response
QIYAGIKVK	42.9	10^{-7}M
QVPLRPMTYK	27.0	10^{-6}M
FPVRPQVPLR	7.8	10^{-7}M
IPRRIRQGL	9.3	10^{-9}M

MM27

81 and 299 DFSOx combined



Epitope peptide	Relative immunodominance of epitope-specific response in early infection (%)	Concentration of peptide required to stimulate half maximum response
KYTAFTIPSI	50.6	10^{-9}M
TPGPGIRYPL	16.3	10^{-8}M

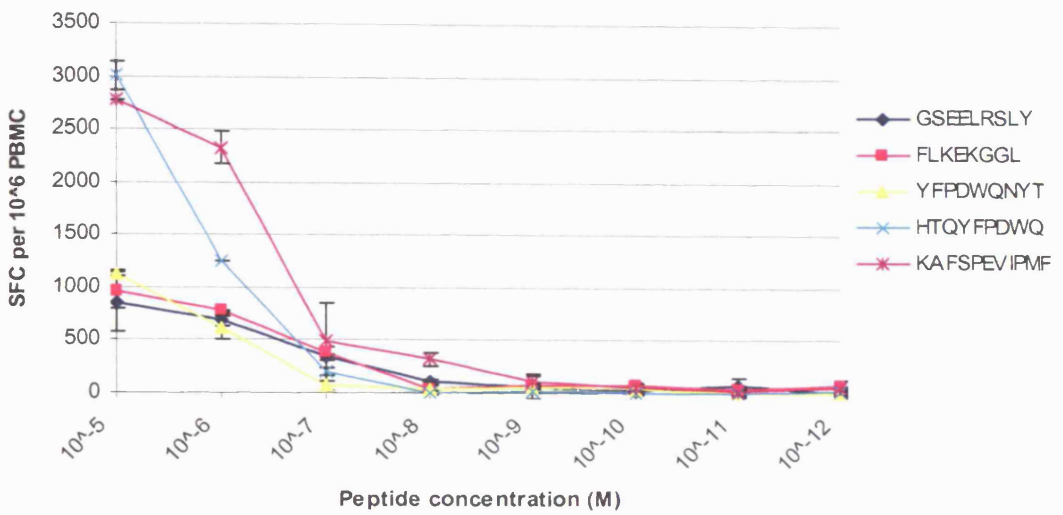


Epitope peptide	Relative immunodominance of epitope-specific response in early infection (%)	Concentration of peptide required to stimulate half maximum response
AAVDLSHFL	11.1	10^{-5}M/10^{-6}M
RGRQKVVSL	4.8	10^{-7}M
GEIYKRWIIL	12.8	10^{-7}M
RRIRQGERALL	53.2	10^{-7}M
FLKEKGGL	11.1	10^{-7}M

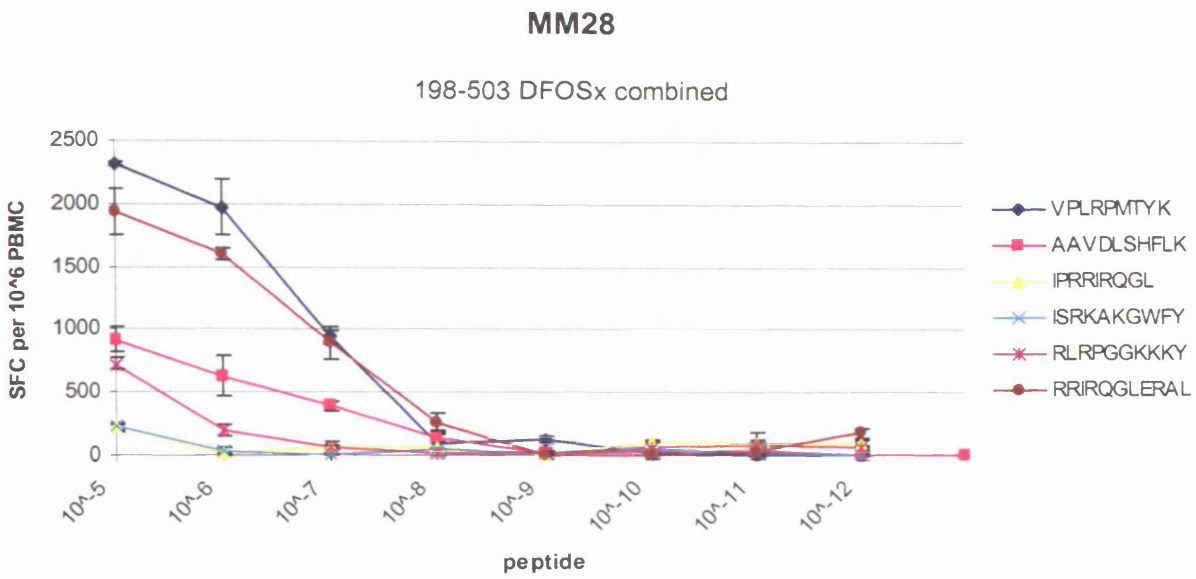
Figure 4.2. Relative immunodominance and avidity of T cell responses made to HIV epitope peptides by patients with intermediate-high persisting viral loads.
In chapter 3, HIV-1 peptides against which $CD8^+$ T cell responses could be detected during early infection in patients MM12, MM27 and MM14 were identified, and the relative immunodominance of the response to each peptide was determined by IFN-γ ELISPOT assay (data in Figures 3.12(a) (MM12), 3.12(c) (MM27) and 3.12(d) (MM14)). The optimal epitopes recognised within some of the peptides against which responses were directed were also identified. In the uppermost panel of each page, the relative avidity of the T cell response to each of these epitopes was determined, by stimulating patient PBMC (cryopreserved at the timepoints indicated) with peptide concentrations between 10^{-5}M and 10^{-12}M, and determining the magnitude of the response elicited by IFN-γ ELISPOT assay. The results shown are expressed as the mean (of duplicate wells) number of specific SFC per 10^6 PBMC (i.e. background values from medium only wells have been subtracted) ±1 SD. The table in the lowermost panel of each page summarises the relative immunodominance of the response to each epitope peptide in early infection (i.e. magnitude of the response to this epitope expressed as a percentage of the total response to all peptides to which responses were detected in this patient) and the relative avidity of each response (concentration of peptide required to stimulate a half-maximal response, calculated from the results of the peptide titrations).

MM13

275 and 353 DFOSx combined



Epitope peptide	Relative immunodominance of epitope-specific response in early infection (%)	Concentration of peptide required to stimulate half maximum response
GSEELRSLY	8.9	10^{-7}
FLKEKGGL	25.2	10^{-7}
YFPDWQNYT	20.7	10^{-6}
KAFSPEVIPMF	9.7	10^{-7}
HTQYFPDWQ	20.7	10^{-6}



Epitope peptide	Relative immunodominance of epitope-specific response in early infection (%)	Concentration of peptide required to stimulate half maximum response
VPLRPMTYK	44.7	10^{-7}M
AAVDLSHFLK	44.7	10^{-7}M
IPRRIRQGL	12.4	$10^{-5/-6}$M
ISRKAKGWFY	7.2	$10^{-5/-6}$M
RLRPGGKKKY	7.5	10^{-6}M
RRIRQGLERAL	12.4	10^{-7}M

Figure 4.3. Relative immunodominance and avidity of T cell responses made to HIV epitope peptides by patients with low-intermediate persisting viral loads.
In chapter 3, HIV-1 peptides against which $CD8^+$ T cell responses could be detected during early infection in patients MM13 and MM28 were identified, and the relative immunodominance of the response to each peptide was determined by IFN-γ ELISPOT assay (data in Figures 3.13(b) (MM13) and 3.13(c) (MM28)). The optimal epitopes recognised within some of the peptides against which responses were directed were also identified. In the uppermost panel of each page, the relative avidity of the T cell response to each of these epitopes was determined, by stimulating patient PBMC (cryopreserved at the timepoints indicated) with peptide concentrations between 10^{-5}M and 10^{-12}M, and determining the magnitude of the response elicited by IFN-γ ELISPOT assay. The results shown are expressed as the mean (of duplicate wells) number of specific SFC per 10^6 PBMC (i.e. background values from medium only wells have been subtracted) ±1 SD. The table in the lowermost panel of each page summarises the relative immunodominance of the response to each epitope peptide in early infection (i.e. magnitude of the response to this epitope expressed as a percentage of the total response to all peptides to which responses were detected in this patient) and the relative avidity of each response (concentration of peptide required to stimulate a half-maximal response, calculated from the results of the peptide titrations).

patient's early HIV-specific T cell response (determined by calculating the magnitude of the response to the epitopic region containing the epitope as a percentage of the total response to all peptides to which responses were detected in the patient) is also shown.

The results of this analysis show that there is not an absolute relationship between functional avidity and relative immunodominance. For example whilst for the two responses studied in patient MM27, the higher avidity response was also the more dominant of the two (the response which required only 10^{-9}M peptide to stimulate half the maximal response constituted over 50% of the entire early response whilst the one with a functional avidity of 10^{-8}M constituted only 16%), the same pattern was not observed for responses studied in other patients, e.g. the highest avidity response (10^{-10}M) in patient MM24 was the least dominant response (13%) of the three studied. Analysis of the relationship between functional avidity and relative dominance of all the responses studied in the seven patients in which this analysis was carried out revealed that there was a trend towards a positive correlation between the functional avidity and relative immunodominance of responses in early HIV infection, although this was not statistically significant ($p=0.139$) (Figure 4.4). Comparison of the functional avidities of the most dominant responses (those individual responses which constituted >25% of the entire early response) with those of the more subdominant responses (Figure 4.5) revealed that there was a trend towards the dominant responses being of higher avidity, although this was not statistically significant ($p=0.149$). Notably however, only 1 of the 9 highly dominant responses had a functional avidity of $<10^{-7}$M, whereas 11 of the 25 more subdominant responses had functional avidities which were $<10^{-7}$M. Hence having a moderate or high functional avidity appears to be almost a pre-requisite for a response to become highly immunodominant, although not all high avidity responses are immunodominant.

To see if the avidity-dominance relationship was any more apparent in patients with higher or lower persisting viral loads, the relative avidities of all dominant responses in the different patient groups were plotted separately. As shown in Figure 4.6, there was a trend towards the dominant responses in the patients with high persisting viral loads being of higher functional avidity than those in the other patients; statistical analysis showed this difference not to be of significance ($p>0.05$) (likely because the number of responses studied in each patient group was very low).

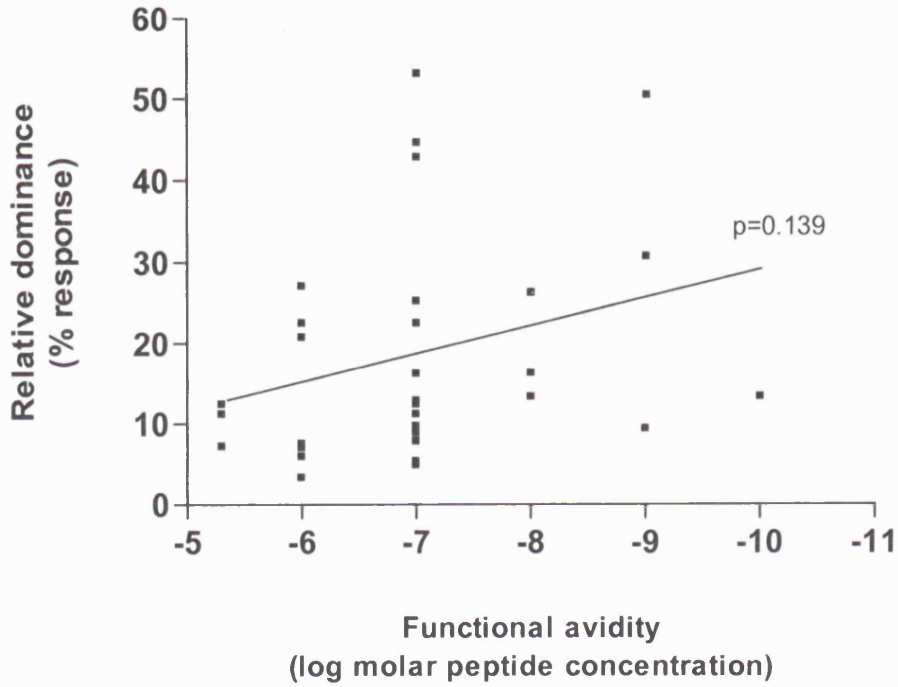


Figure 4.4. Relationship between the functional avidity and relative dominance of HIV-specific $CD8^+$ T cell responses. The functional avidity of selected epitope-specific responses found to be part of the early response of seven HIV-1 infected patients was determined as described in Figures 4.1-4.3. The relative dominance of each response was defined as the magnitude of the response made to the epitope–containing long peptide (determined in experiments described in section 3.5) as a proportion of the total magnitude of the early response (i.e. that to all epitopic regions found to be recognised by the patient). The functional avidities (expressed as the log concentration of epitope peptide required to simulate a half maximal response by early patient PBMC) and relative immunodominance (expressed as a percentage of the total early response) of epitope-specific responses mapped in patients MM24, MM26, MM12, MM27, MM14, MM13 and MM28 are plotted above. A linear regression line is shown and the p value shown was calculated using Pearson's correlation test.

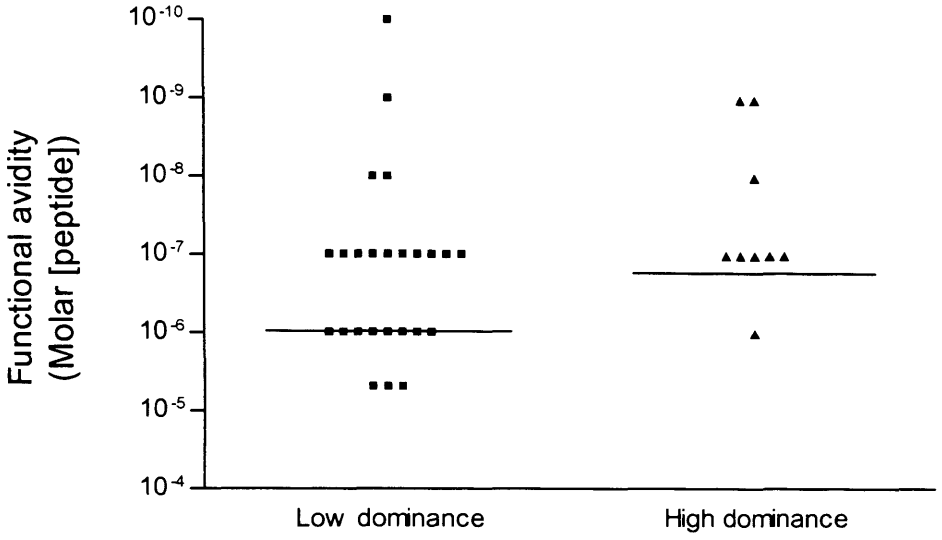


Figure 4.5. Comparison of the functional avidity of responses of relative high and low dominance. The functional avidity and relative dominance in early infection of selected epitope-specific responses mapped in seven HIV-infected subjects (MM24, MM26, MM12, MM27, MM14, MM13 and MM28) was determined as described in Figures 4.1-4.3. Each symbol represents the functional avidity of an individual epitope-specific response. Responses were split into two groups on the basis of the percentage of the total response (i.e. that to all epitopic regions found to be recognised by the patient) constituted by the response to the epitope-containing long peptide being considered. Responses were considered of high dominance if they contributed to greater than 25% of the total response; all other responses were considered to be of low dominance. The mean functional avidity of each group of responses is indicated by the horizontal bar. Statistical analysis (a two-tailed t-test) showed that there was not a significant difference between the functional avidities of responses of low and high dominance.

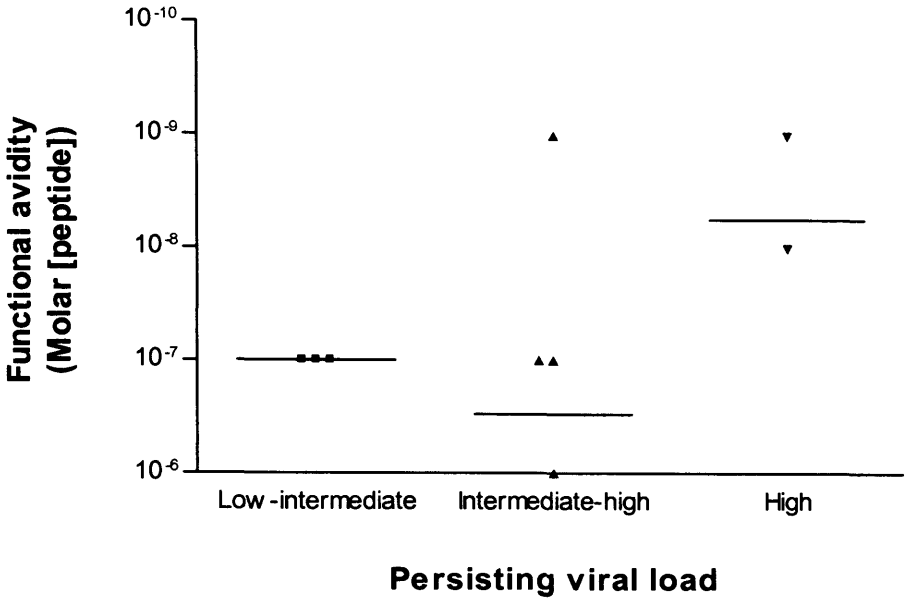


Figure 4.6. Comparison of the functional avidities of dominant responses in patients with different persisting viral loads. The functional avidity and dominance of selected epitope-specific responses found to be part of the early HIV-specific $CD8^+$ T cell response in seven HIV-infected subjects were determined as described in the legend in Figures 4.1-4.3. Individual responses which constituted over 25% of a patient's entire early response were considered to be highly dominant. The avidities of these dominant responses are shown above and have been grouped on the basis of the persisting viral load established by the patient in which the epitope was mapped. This analysis was carried out for patients MM24 and MM26 (patients with high persisting viral loads); MM12, MM27 and MM14 (patients with intermediate-high persisting viral loads) and MM13 and MM28 (patients with low-intermediate persisting viral loads). Each data point represents the avidity of an individual dominant response, with the mean avidity of the dominant responses in each patient group indicated by the horizontal line. Statistical analysis (one-way ANOVA) showed that there was not a significant difference between the functional avidities of dominant responses in patients with different persisting viral loads.

It should be mentioned that this data may be affected by the fact that some epitope-specific responses within a patient's whole response were not represented in the analysis (the optimal epitopes were not mapped for all epitopic regions within a patient's entire early response), and if/how they would have made any difference to the overall picture is not known. Also the functional avidities were not determined at precisely the same timepoints at which the relative magnitudes of the responses to the different epitopic regions were measured. These limitations are considered further in the discussion.

In summary, in this investigation of the relationship between the relative avidity and dominance of HIV-specific responses in early infection, an absolute relationship between the two parameters was not found. However, there was a weak positive association, with dominant responses tending to have a higher functional avidity than subdominant responses and very few dominant responses being of low avidity. The relationship may also be more apparent in particular patients, since the dominant responses in patients with higher persisting viral loads tended to be of higher functional avidity. These data suggest that the avidity of a response is one of the factors that influences its immunodominance. The relationship is not absolute however, and so other factors (such as repertoire-related factors) must also contribute to the immunodominance of responses.

4.3 Analysis of the effect of lack of $CD4^+$ T cell help at the time of priming of a virus-specific $CD8^+$ T cell response on the epitope hierarchy of the response induced

The analysis above provided evidence for a weak association between the relative immunodominance and functional avidity of responses in early HIV-1 infection. However, because there were not many patients with lower persisting viral loads available for comparison, the question of whether there was a greater dominance of high avidity responses in patients with the highest viral loads could not be fully addressed. Even had a full analysis been feasible, and a difference observed, this would not have revealed anything about the mechanisms involved. Because of the limitations of the human studies, murine models were used to assess more directly the question of whether defects in T cell help/APC activation at the time of induction of a virus-specific immune response leads to preferential expansion of high avidity responses.

The $CD8^+$ T cell response made to LCMV in H-2^b mice was the first model used. In this infection, the virus induces a high frequency $CD8^+$ T cell response to a number of well defined epitopes in a reproducible hierarchy (Murali-Krishna *et al.*, 1998; van der Most *et al.*, 2003; van der Most *et al.*, 1998). The sequences of the principal epitopes (4 D^b and 2 K^b restricted epitopes) known to be targeted by the LCMV-specific $CD8^+$ T cell response are shown in Table 4.1. As summarised in this table, prior studies addressing the relative immunodominance of the response to these epitopes have indicated that the epitopes to which the highest magnitude responses are mounted in primary LCMV infection are the NP396 and GP33 epitopes, with the responses to other epitopes being of lower magnitude, and the response to the GP92 epitope being particularly subdominant. The binding affinity of these epitopes to their MHC restriction elements has also previously been determined; the epitope which has the highest affinity binding for its MHC restriction element is NP396 (Table 4.1). The NP396-specific response also has the highest functional avidity (Table 4.1). The data indicate that, with the exception of the GP33 epitope, there is a good correlation between the affinity and immunodominance of epitopes targeted in natural LCMV infection with a very high affinity epitope being particularly prominent in the virus-specific T cell response. The question of whether there would be greater bias towards the highest affinity response(s) if help was limiting at the time the response was induced was addressed.

CD4 help for the expansion of the LCMV-specific $CD8^+$ T cell response was removed by the administration of a CD4-depleting antibody to mice just prior to infection with LCMV. CD4-depleted and control, non-depleted mice were infected with LCMV, and splenocytes were taken at day 8 after infection (when expansion of virus-specific cells is maximal), and the response to different LCMV epitopes was measured by IFN-γ ELISPOT assay. The responses to only five peptides were tested since, due to the overlap between the GP33 and GP34 epitopes, only the response to the GP33 (9mer) sequence, and not the response to the GP34 epitope, was tested. Figure 4.7 shows the average responses made to the five LCMV epitope peptides tested by mice in the two experimental groups. In the control mice, the GP33, NP396 and NP205 peptides all stimulated high magnitude responses, in excess of 20,000 SFC per 10^6 $CD8^+$ cells; the GP276-specific response was more subdominant, and the GP92-specific response very minor by comparison. It was noteworthy that the hierarchy of the response was not the same as that previously reported by

Epitope	Sequence	MHC restriction	Relative immunodominance in primary response (% $CD8^+$ T cells)			MHC binding affinity IC_{50} [nM] (iii) / SC_{50} [nM] (iv)						Functional avidity [j] ED_{50} [nM]
			c (i)	d (i)	d (ii)	e (iii)	f (iii)	g (iii)	g (iv)	h (iii)	h (iv)	
NP396	FQPQNGQFI	D^b	28	19	8.7	4.4	N.D.	20	50	10	7	0.0002
GP33	KAVYNFATC(GI) [a]	D^b	19	20	8.3	5429 (776)	5429 (395)	615	344	21 (51)	470 (477)	0.26
GP276	SGVENPGGYCL	D^b	7	5.6	1.9	52	N.D.	37	42	26	51	1.9
NP205	YTVKYPNL	K^b	8	5.9	3.2	170	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
GP92	CSANNSHHYI	D^b	0.7	0.5	N.D.	470	N.D.	N.D.	N.D.	9	44	N.D.
GP34	AVYNFATCGI	K^b	N.D.	N.D.	4.8	N.D.	22	N.D.	N.D.	N.D.	N.D.	N.D.

Table 4.1 LCMV $CD8^+$ T cell epitopes in H-2^b mice. The sequence, MHC restriction, proportion of the response elicited, relative affinity of binding to MHC and functional avidity of six epitopes targeted by the virus-specific $CD8^+$ T cell response in primary LCMV infection of H-2^b mice are given in the table above. Data from more than one study are given for comparison in some cases.
N.D. means that measurements for these peptides were not determined.

[a] Two possible sequences, a 9-mer and an 11-mer, have been shown to be the optimal epitope, the results varying on the method used to evaluate the response.

[c] Data from (Murali-Krishna *et al.*, 1998)
[d] Data from (van der Most *et al.*, 2003)
(i) intracellular IFN-γ staining (ii) tetramer staining

[e] Data from (van der Most *et al.*, 1998)
[f] Data from (Puglielli *et al.*, 2001)
[g] Data from (Gairin *et al.*, 1995)
[h] Data from (Hudrisier *et al.*, 1996)
(iii) competition assay: IC_{50} - concentration of competitor peptide that inhibits 50% of the specific binding of bound radio-labelled probe on T2 cells.
(iv) stabilization assay: SC_{50} - concentration of peptide that induces half maximal stabilization of H-$2D^b$ on RMA-S cells incubated at 37°C in the presence of increasing concentrations of peptide.

[j] Data from (Gairin *et al.*, 1995). Cytotoxicity assay: ED_{50} - peptide concentration required to induce half maximal lysis of radio-labelled peptide-pulsed target cells by LCMV-specific CTL clones/bulk splenocytes.

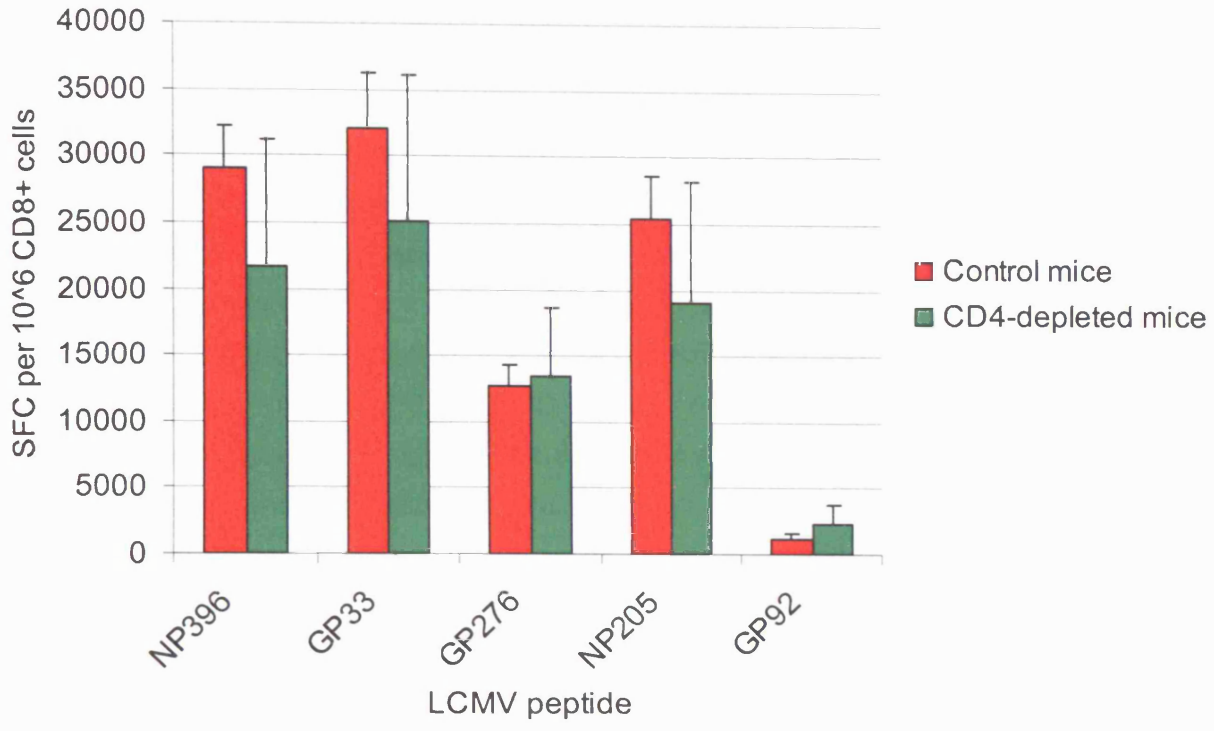


Figure 4.7. Analysis of the magnitude of $CD8^+$ T cell responses to different LCMV epitopes in $CD4^+$ cell-depleted mice and control C57BL/6 mice infected with LCMV. Control and CD4-depleted C57BL/6 mice were infected i.p. with $2x10^5$ pfu LCMV Armstrong. The CD4-depleted mice were given 1mg of anti-CD4 monoclonal antibody GK1.5 i.v. one day prior to and then 2 days after LCMV infection to remove CD4 T cell help for the induction of the LCMV-specific $CD8^+$ T cell response. 8 days later the frequencies of splenic $CD8^+$ T cells specific for different LCMV epitopes were determined by incubating splenocytes with synthetic peptides corresponding to LCMV $CD8^+$ T cell epitopes (final concentration 10^{-6} M), and measuring the number of cells stimulated to produce IFN-γ by ELISPOT assay. The percentage of $CD8^+$ T cells in each splenocyte preparation was also analysed (by antibody staining and FACS), so that epitope-specific cell frequencies within the $CD8^+$ T cell compartment could be calculated. The data shown represent the mean of results obtained in groups of four mice, and are expressed as the average number of specific spot forming cells (SFC) per 10^6 $CD8^+$ T cells (i.e. with background values from medium only wells subtracted), +1 SE. Staining of splenocyte samples with an antibody against CD4 showed that the depletion had reduced the number of splenic $CD4^+$ cells to <0.3% of the number found in control mice. This is one representative example of three experiments carried out, which all gave similar results.

other groups (Murali-Krishna *et al.*, 1998; van der Most *et al.*, 2003; van der Most *et al.*, 1998): the strongest response was that towards the GP33 peptide, and a larger than expected response was made to the normally subdominant NP205 epitope. Potential reasons for these discrepancies are considered in the discussion. In the CD4-depleted mice, the overall magnitude of the response was slightly reduced compared to the control group, but nonetheless, a strong response was still seen. In the representative experiment depicted in Figure 4.7, although the magnitude of the responses to the three most dominant epitopes was diminished in CD4-depleted mice, the responses to the more subdominant epitopes appeared to be preserved. Importantly however, CD4 depletion did not induce a major change in the immunodominance hierarchy: responses were still ranked in the order GP33>NP396>NP205>GP276>GP92. Thus there was no evidence in this experimental system to suggest that there was more of a bias to high affinity epitopes when the CD8 response was induced in the absence of CD4 help.

Previous studies have shown that in LCMV-infected mice, the widespread virus replication in lymphoid tissues can substitute for $CD4^+$ T cell help in the induction of the virus-specific $CD8^+$ T cell response (Christensen *et al.*, 1994; Janssen *et al.*, 2003; Leist *et al.*, 1987; Moskophidis *et al.*, 1987b); in line with this, CD4 depletion had only a minor effect on the magnitude of the CD8 response induced following infection of mice with LCMV in the experiment described above (a two-tailed t-test showed that the total magnitudes of the LCMV-specific responses were not significantly different between mice in the two groups). Therefore LCMV infection of CD4-depleted mice was perhaps not the most suitable model to use to mimic the limited availability of fully activated APCs hypothesised to be present in primary HIV infection (where, unlike in this model of acute LCMV infection, there are thought to be direct effects of the virus on APC function in addition to indirect effects due to limited availability of CD4 help). It was thus decided to use infection of mice with recombinant vaccinia viruses encoding the NP and GP genes of LCMV Armstrong (VV-NP and VV-GP) as an alternative model system. Here, responses to the LCMV epitopes could still be studied; however, because vaccinia virus (unlike LCMV) does not replicate extensively in lymphoid tissues, it was hoped that there would be less infection-associated APC activation, and so CD4 help would be more critical for induction of the virus-specific $CD8^+$ T cell response in this situation.

Control C57BL/6 mice and IA k/o mice which lack MHC class II-restricted CD4 helper T cells (because their APCs do not express MHC class II molecules) were infected with a mixture of VV-NP and VV-GP, and eight days later the splenic LCMV-specific CD8 response was assessed using an IFN-γ ELISPOT assay. IA k/o mice were used rather than CD4 depleted mice, as this was a better way of ensuring that the mice were completely CD4 cell deficient.

In Figure 4.8 it can be seen that the magnitude of the overall response to the LCMV epitopes was approximately 10-fold lower in the recombinant vaccinia-LCMV-infected mice than that seen in LCMV-infected mice in Figure 4.7. Similar observations have been made in previous studies (Hassett *et al.*, 2000; von Herrath *et al.*, 1996); the difference is thought to be due in large part to the relative amounts of LCMV antigen generated in the two infections.

In the control mice, the GP33-specific response was clearly the most dominant in this experiment; the GP276 and NP396-specific responses were the next most dominant, followed by a small response to NP205, and the response to GP92 was below the limit of detection of the assay. In the IA k/o mice, responses were seen to the same four epitopes, with the magnitude of the response being diminished compared to the control mice for all but the high affinity NP396 epitope, the response to which was actually higher than that in control mice (however, this difference was not statistically significant as judged by a two-tailed t-test ($p=0.246$), which may be due to the small sample size in the experiment). The hierarchy of the response immunodominance in control and IA k/o mice thus differed: in the control mice it was in the order GP33 > GP276 $\approx$NP396 > NP205 > GP92, whereas in the IA k/o mice it was GP33 $\approx$ NP296 > GP276 $\approx$NP205 > GP92. These results lend some support to the idea that there may be better priming of the response to the highest affinity epitope when CD4 help is absent.

Given that relatively high magnitude responses were still raised against the recombinant vaccinia-LCMV viruses in the IA k/o mice, the virus-specific CD8 response in this infection also appeared to be largely independent of CD4 help (again, statistical analysis (a two-tailed t-test) showed that the total magnitudes of the virus-specific responses were not significantly different for mice in the two groups). A further series of experiments were thus carried out using a different model, involving infection of mice with influenza virus via the i.p. route. Productive infection by influenza A virus is essentially limited to the lung because a protease required to cleave the influenza virus haemagglutinin molecule is restricted in distribution to the respiratory epithelium (Rott *et al.*,

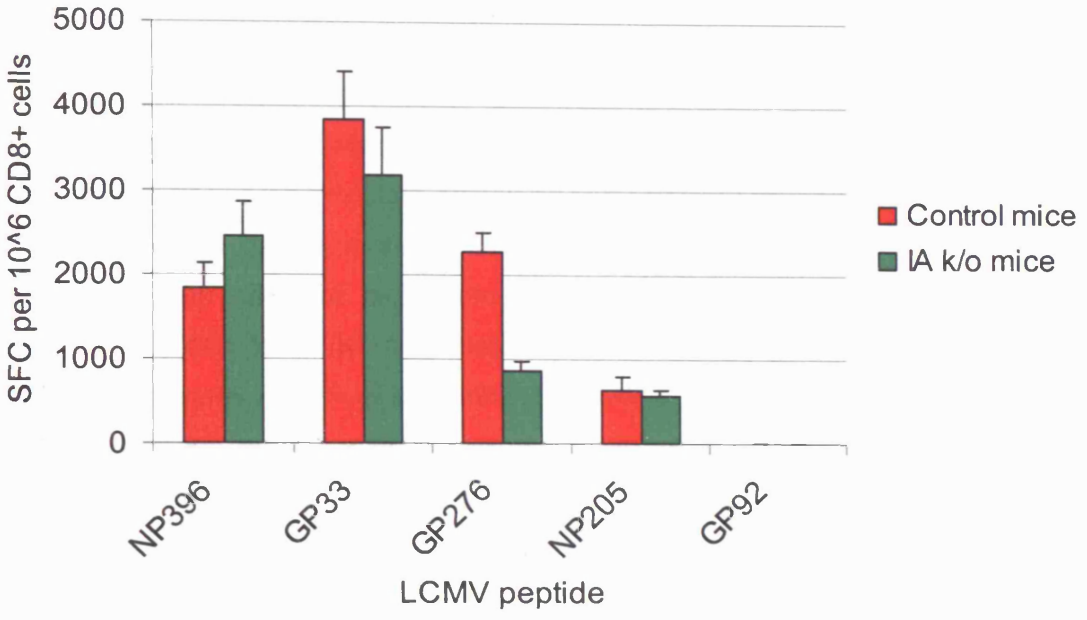


Figure 4.8. Analysis of the magnitude of $CD8^+$ T cell responses to different LCMV epitopes in IA k/o mice and control C57BL/6 mice infected with VV-LCMV. Control and IA k/o mice were infected i.p. with a mixture of two recombinant vaccinia viruses: one expressing the NP gene of LCMV Armstrong and the other expressing the GP gene (10^6 pfu of each virus used). 8 days later, mice were culled and the frequencies of splenic $CD8^+$ T cells specific for different LCMV epitopes were determined by incubating splenocytes with synthetic peptides corresponding to LCMV $CD8^+$ T cell epitopes (final concentration 10^{-6} M), and measuring the number of cells stimulated to produce IFN-γ by ELISPOT assay. The percentage of $CD8^+$ T cells in each splenocyte preparation was also analysed (by antibody staining and FACS), so that epitope-specific cell frequencies within the $CD8^+$ T cell compartment could be calculated. The data shown represent the mean of results obtained in groups of four mice, and are expressed as the average number of specific spot forming cells (SFC) per 10^6 $CD8^+$ T cells (i.e. with background values from medium only wells subtracted), +1 SE. These results are representative of two independent experiments, in which similar results were obtained.

1995; Walker *et al.*, 1992). By delivering the virus by an i.p. route, viral antigens can be presented but without virus replication occurring. It was hoped that induction of a CD8 T cell response to viral epitopes would be more dependent on CD4 help in this system.

Influenza A virus is a negative-stranded RNA virus whose genome consists of 8 RNA segments that encode 11 protein products, the majority of which serve as targets for T cell recognition. A number of H-2^b-restricted $CD8^+$ T cell epitopes have been defined for influenza (Table 4.2), but the relative immunodominance and relative affinity of MHC binding is not known for all of the epitopes. To gain a more complete picture of the relative MHC binding affinities of the different H-2^b-restricted influenza epitopes, binding of the epitope peptides to D^b/K^b was determined experimentally using an MHC stabilisation assay (Gairin *et al.*, 1995; Hudrisier *et al.*, 1996). RMA-S cells are a TAP-deficient mutant cell line which have empty MHC molecules that accumulate on the cell surface when the cells are grown at 26°C, but are unstable and disappear from the cell surface when the cells are incubated at 37°C. Surface expression of MHC molecules can be stabilised by addition of peptides that bind to the empty MHC molecules. The ability of a peptide to stabilise MHC molecules on the cell surface correlates with the peptide's affinity for the MHC. Stable complexes remain at the surface when the cells are incubated at 37°C and can be detected by staining with fluorescently labelled anti-MHC class I antibodies. By measuring the concentration of an epitope peptide required to stabilise MHC expression on the cell surface for several epitope peptides, their relative binding affinities can be compared.

In Figure 4.9, the stabilisation of K^b expression by K^b-binding influenza epitopes PB1 703, PB2 198, NS2 114, MI 128 and the stabilisation of D^b expression by D^b-binding influenza epitopes PA 224, NP 366, DAMP 62 is shown, together with that of positive control peptides known to bind with high affinity to K^b/D^b. Three peptides (PB2 198, NS2 114 and PA 224) stabilised moderate/high levels of MHC expression in a dose-dependent fashion. The fluorescence data obtained was used to calculate pBL_{50} values (the negative log of the peptide concentration yielding the half-maximal fluorescence intensity of the reference peptide) for each peptide; the pBL_{50} values for PB2 198, NS2 114 and PA 224 were -7.6, -2.6 and -13.3, respectively, indicating that that PA224 had a particularly high MHC binding affinity. The other influenza peptides did not exhibit dose-dependent stabilisation of sufficiently high levels of MHC expression to enable pBL_{50} values to be calculated,

Epitope	Sequence	MHC restriction	Immunodominance rank [a]	MHC binding affinity		
				SD_{50} value (nM) [b]	IC_{50} value (nM) [c]	pBL_{50} value [d]
PA 224	SSLENFRAYV	D^b	1	72.2	n.d.	-13.3
NP 366	ASNENMETM	D^b	2	15.2	24	b.n.d.
DAMP 62	LSLRNPILV	D^b	3	n.d.	n.d.	n.d.
PB1 703	SSYRRPVGI	K^b	4	47.4	n.d.	b.n.d.
PB2 198	ISPLMVAYM	K^b	5	n.d.	n.d.	-7.6
NS2 114	RTFSFQLI	K^b	6	500-15000	9.7	-2.6
M1 128	MGLIYNRM	K^b	7	188.3	4.6	b.n.d.

Table 4.2. Peptides in Influenza A PR8 virus recognised by virus-specific $CD8^+$ T cells in H-2^b mice. The sequence, MHC restriction, rank according to the magnitude of the CD8 response elicited to the epitope in primary influenza infection in H-2^b mice and relative affinity of binding to MHC of seven peptides recognised by influenza virus-specific $CD8^+$ T cells are given in the table above. n.d. means that affinity measurements for these peptides were not determined. b.n.d. means that binding was not detected.

a. Data from (Chen *et al.*, 2002)
b. Data from (Zhong *et al.*, 2003)
c. Data from (Vitello *et al.*, 1996)
d. Calculated from data in Figure 4.9

Explanations for calculation of SD_{50} and IC_{50} values are given in Table 4.1; pBL_{50} values were calculated as explained in the text.

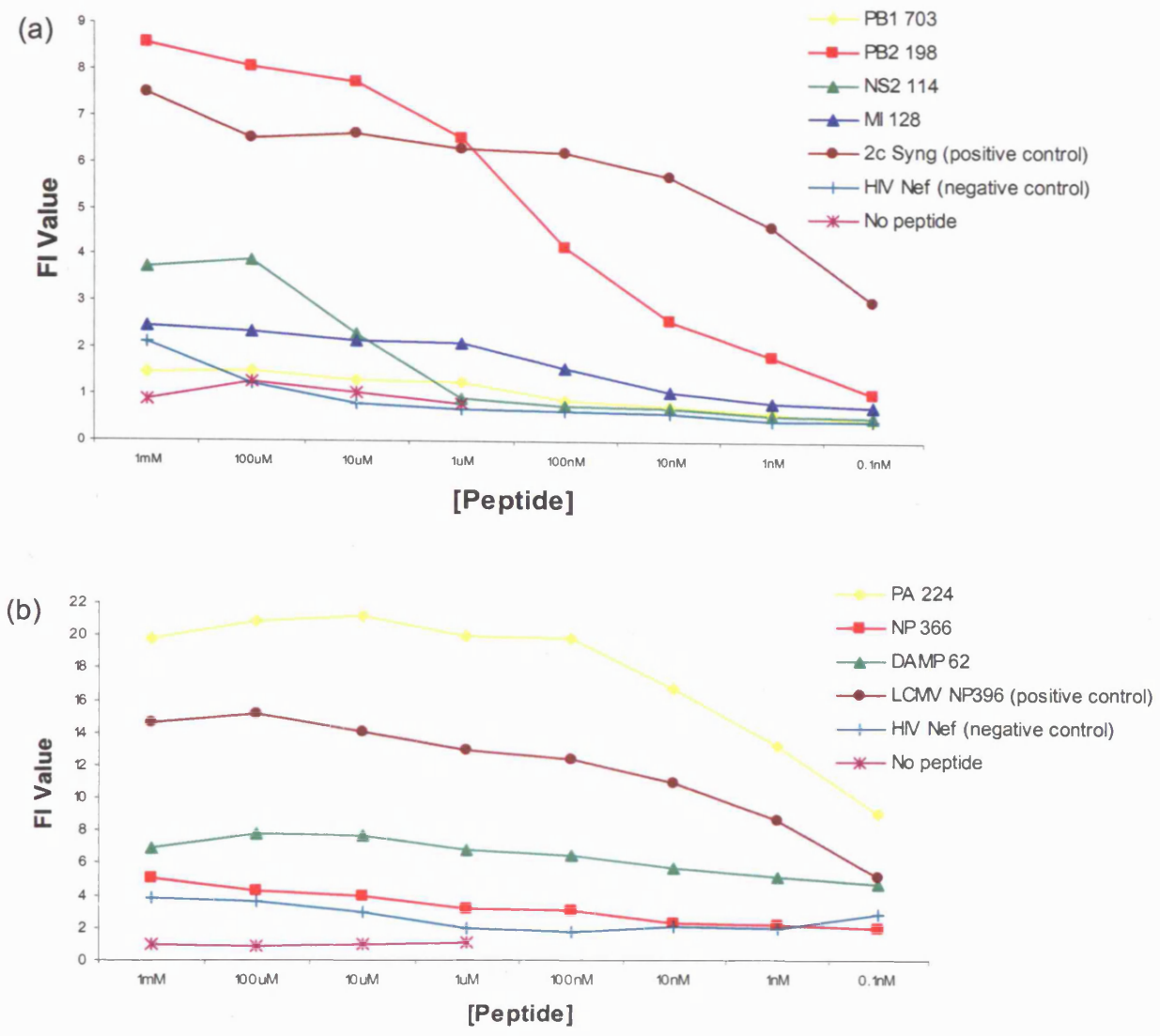


Figure 4.9. Determination of the relative binding affinities of influenza epitope peptides to H-2 alleles using a MHC stabilisation assay. RMA-S cells were cultured at 37°C in the presence of a series of dilutions (1mM to 0.1nM) of peptides corresponding to influenza epitopes. The relative affinity of binding of the peptides to MHC was assessed by their ability to stabilise expression of K^b or D^b molecules on the cell surface, and was measured by staining the cells with FITC-conjugated anti-class I H-2K^b or H-2D^b (or isotope control) antibodies. The mean fluorescence intensity (MFI) of each sample was determined by flow cytometry and results are expressed as fluorescence index (FI) values [FI = (MFI of test peptide - MFI of no peptide isotype control)/ (MFI of no-peptide class I -stained control - MFI of no peptide isotype control)]. The stabilisation of K^b expression by K^b-binding influenza epitopes PB1 703, PB2 198, NS2 114, M1 128 is shown in (a), and the stabilisation of D^b expression by D^b-binding influenza epitopes PA 224, NP 366, DAMP 62 is shown in (b). No peptide, positive (2c Syng for K^b assay and LCMV NP396 for D^b assay) and negative (HIV Nef $_{82\text{-}90}$) control peptides were also included in the assay. These data are representative of results obtained in two independent experiments.

suggesting that their MHC binding affinities were too low to be assessed by this approach. As indicated in Table 4.2, there are discrepancies among the results obtained by other groups who have addressed the affinity of binding of these influenza virus peptides to MHC, and between the results obtained here and the different sets of published data; possible reasons for this are discussed later.

The hierarchy of responses induced to this panel of influenza virus epitope peptides when control and IA k/o mice were infected i.p. with influenza PR8 virus was then investigated. Mice were sacrificed on day 8 post-infection, and the epitope-specificity of splenic $CD8^+$ cells assessed in an IFN-γ ELISPOT assay. In Figure 4.10 it can be seen that the control mice made dominant responses to the NP 366, PB2 198 and PA 224 epitopes, with the response to DAMP 62 being of slightly lower magnitude, and then smaller responses being made to the NS2 114, M1 128 and PB1 703 epitopes. In the IA k/o mice responses to all peptides were lower in magnitude than those in the control mice, and the hierarchy of the responses was slightly different; but there was no evidence to support the idea that there may be preferential induction of responses to high affinity epitopes when CD4 help was absent. However it was notable that despite efforts to choose a system which it was thought might be more dependent on help for priming a virus-specific $CD8^+$ T cell response, the IA k/o mice were still able to make reasonable responses to the virus (once again, the total magnitudes of the virus-specific responses were not significantly different for mice in the two groups, as judged by a two-tailed t-test). Hence again, it was not possible to rigorously address the effects of incomplete APC activation on the hierarchy of responses primed to different viral epitopes using this system.

4.4 Analysis of the effect of lack of $CD4^+$ T cell help at the time of priming of a virus-specific $CD8^+$ T cell response on the clonality of the response induced

In experiments described above, the absence of $CD4^+$ T cell help at the time of priming of a virus-specific response was not found to have a great influence on the epitope hierarchy of the $CD8^+$ T cell response induced. However it was possible that alterations were induced in the clonal composition of the response (e.g. there may be selective expansion of T cells with high affinity TCRs). To gain an overview of the TCR usage by virus-specific T cells induced in control and CD4-depleted mice infected with LCMV, TCR Vβ family

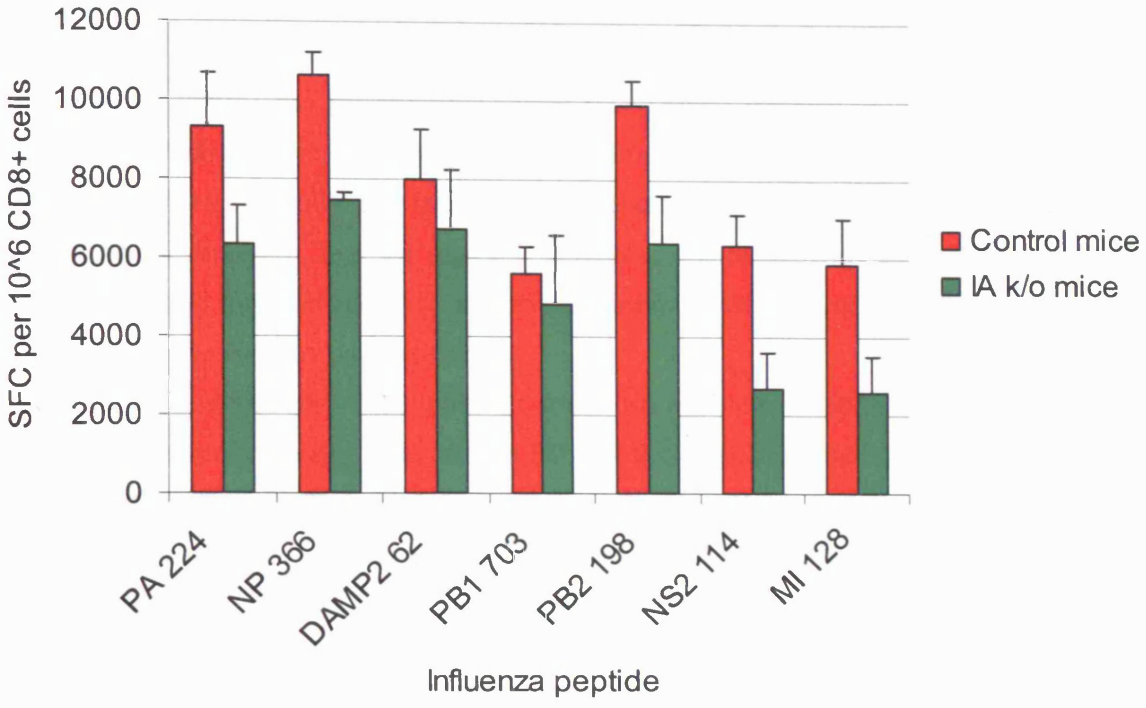


Figure 4.10. Analysis of the magnitude of $CD8^+$ T cell responses to different influenza epitopes in IA k/o mice and control C57BL/6 mice infected with influenza virus. Control and IA k/o mice were infected with 600 hemagglutinating units of influenza virus PR8 i.p. Eight days later, the mice were culled and the frequencies of splenic $CD8^+$ T cells specific for different influenza epitopes were determined by incubating splenocytes with synthetic peptides corresponding to influenza $CD8^+$ T cell epitopes (final concentration 10^{-6} M), and measuring the number of cells stimulated to produce IFN-γ by ELISPOT assay. The percentage of $CD8^+$ T cells in each splenocyte preparation was also analysed (by antibody staining and FACS), so that epitope-specific cell frequencies within the $CD8^+$ T cell compartment could be calculated. The data shown represent the mean of results obtained in groups of four mice, and are expressed as the average number of specific spot forming cells (SFC) per 10^6 $CD8^+$ T cells (i.e. with background values from medium only wells subtracted), +1SE. This experiment is representative of results obtained in two independent experiments.

usage by cells responding to two LCMV epitopes was assessed by staining with antibodies specific for different TCR Vβ families. Peripheral blood pooled from groups of four control or CD4-depleted LCMV-infected mice (bled on day 8 post-infection) was stained with tetramers of one of two specificities (NP396 or GP33), an anti-CD8 antibody, plus one of a panel of antibodies specific for different TCR Vβ families. The usage of different TCR Vβ families by the antigen-specific cells was then assessed by flow cytometry. The TCR Vβ families used (and proportion of cells using each family), by both the NP396 and GP33-specific cells in the control mice and IA k/o mice are shown in Figure 4.11. Despite the fact that antibodies were not available to detect all TCR Vβ families, the Vβ staining detected all of the antigen-specific cells, suggesting that the panel of antibodies used covered all of the major TCR Vβ families involved in each response.

The NP396-specific CD8 cells in the control mice exhibited very restricted TCR Vβ family usage, with almost 90% of the epitope-specific cells using Vβ8$^+$ or Vβ 8.1/8.2$^+$ TCRs. Slightly more diversity in TCR Vβ use was seen in the GP33-specific population in the control mice, with Vβs 7 and 8.3 being used in addition to Vβ 8 and Vβ 8.1/8.2. TCR Vβ family usage by NP396 and GP33-specific cells from IA k/o mice was very similar to that in control animals. There did not therefore seem to be any major difference in the repertoire of TCR Vβ families used in the LCMV-specific response when CD4 help was limiting. However, as discussed above, APC activation may not have been appreciably compromised in these animals despite the lack of CD4 help.

In summary, the series of experiments described in sections 4.3 and 4.4 showed that deficiency of CD4$^+$ T cell help during an acute virus infection does not dramatically affect the hierarchy of epitope or clonal immunodominance in the primary virus-specific CD8$^+$ T cell response. However the model systems used here did not allow the effects of deficits in APC numbers/activation state in addition to paucity of CD4$^+$ T cell help on the nature of the primary virus-specific CD8 response to be explored properly.

4.5 Search for epitope-specific CD8$^+$ T cell responses induced to viruses other than HIV that are able to cross-recognise HIV epitopes and may rapidly expand in acute HIV infection to become an immunodominant component of the primary HIV-specific CD8$^+$ T cell response

A second potential hypothesis to explain the strong responses made to single (or small numbers of) immunodominant epitopes in some patients undergoing

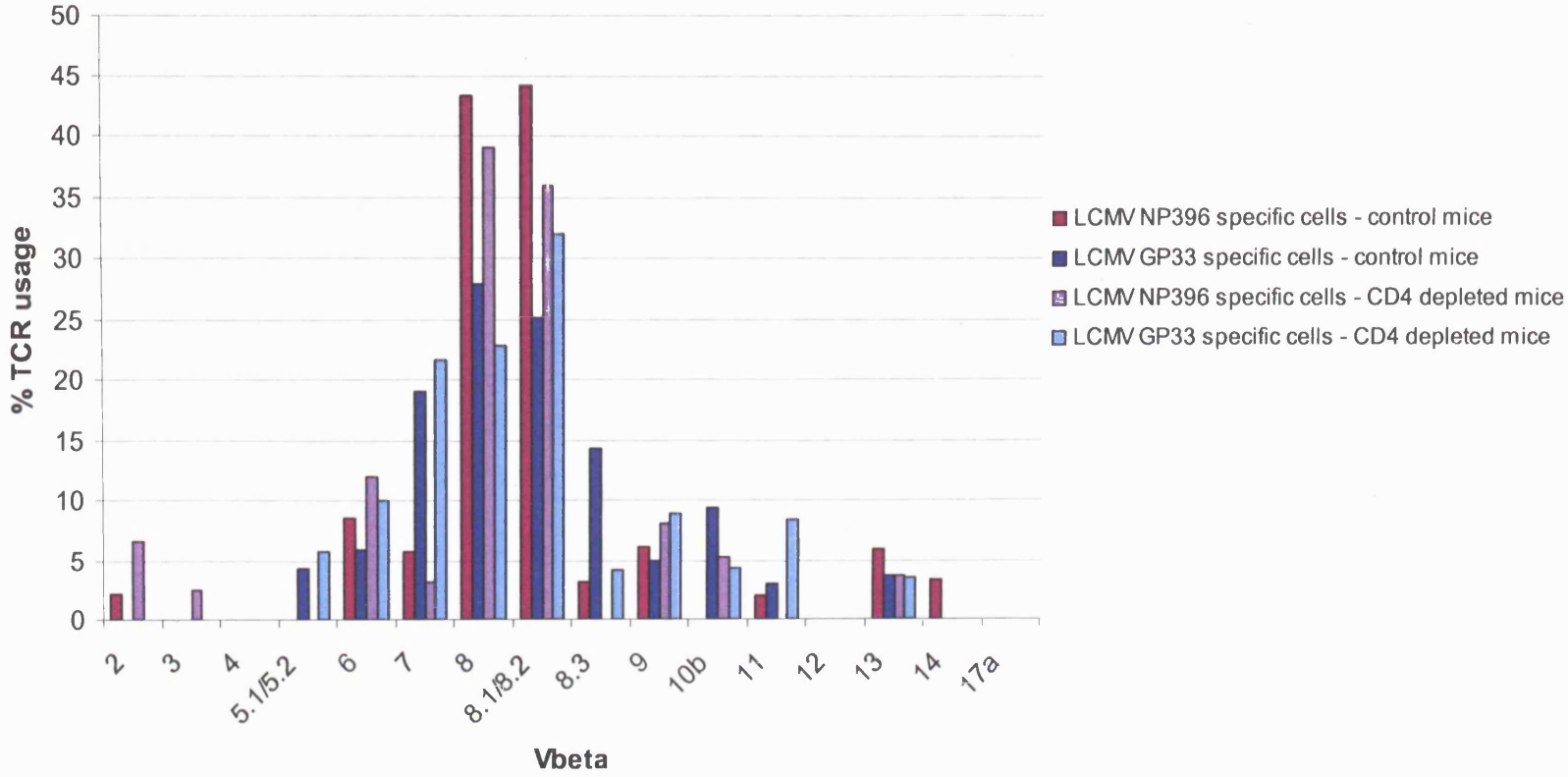


Figure 4.11. Analysis of TCR Vβ family usage by NP396 and GP33-specific peripheral blood T cells in control and CD4-depleted mice acutely infected with LCMV. Control C57BL/6 and CD4-depleted C57BL/6 mice were infected i.p. with $2x10^5$ pfu LCMV Armstrong. Depletion of $CD4^+$ cells was achieved by the administration of 1mg anti-CD4 monoclonal antibody GK1.5 one day prior to and then 2 days after LCMV infection. Eight days later, mice were culled and blood lymphocytes pooled from groups of four mice were co-stained with anti-CD8 mAb, LCMV NP396 or LCMV GP33 tetramer, and a panel of TCR Vβ-specific mAbs. The results shown are the percentage of tetramer positive cells for each epitope stained with each TCR Vβ family-specific mAb in control and CD4-depleted mice. Values less than 2% were regarded as background staining and are not shown on the graph. The efficiency of the CD4 depletion was also checked by staining splenocytes with an anti-CD4 mAb. This showed that the number of splenic $CD4^+$ cells in the depleted mice had been reduced to <0.3% of the number seen in the control mice.

primary HIV-1 infection is that the cells used to make these responses may be cross-reactive memory cells which are triggered to expand upon recognition of HIV antigens more rapidly than naïve cells (due to their high frequency and ease of re-activation). Such cross-reactive memory cells may also be less dependent on help/full APC activation for their expansion than naïve cells.

One example of an epitope-specific response that was particularly immunodominant in primary HIV infection was that made by patient WEAU to a HLA-B44-restricted epitope (AENLWVTVY(Y)) in gp160 (Borrow *et al.*, 1997). During acute infection, >40% of peripheral blood T cells in this patient may have been directed towards this epitope (Pantaleo *et al.*, 1994), whilst responses to other epitopes were very low at this time (Borrow *et al.*, 1997). Did this response arise as a consequence of rapid expansion of memory cells generated as a result of a previous infection that were able to cross-recognise this gp160 epitope? To examine this possibility, potentially cross-reactive epitopes were looked for. An initial search was made for known HLA-B44*03-restricted epitopes in other viruses that had similarity to the HIV immunodominant AENLWVTVY(Y) epitope. EBV was found to have a HLA-B44*03-restricted epitope (EENLLDFVRF) sharing several amino acids with the HIV epitope, and having chemically similar residues at other positions. The ability of peptides corresponding to the two viral epitopes to sensitise autologous target cells for lysis by polyclonal CTL derived from PBMC cryopreserved from WEAU was compared using a ^{51}Cr release assay. The AENLWVTVYY peptide was able to induce around 40% specific lysis, but no lysis of target cells pulsed with the EENLLDFVRF peptide was seen (data not shown - experiment carried out by Dr N. A. Jones). This result suggested that it was unlikely that this EBV epitope had primed the cells that may have later cross-recognised HIV AENLWVTVY(Y).

The possibility of cross-recognition of the immunodominant HIV epitope AENLWVTVYY by individuals who had a response to the EBV epitope EENLLDFVRF was further explored using cells from subject JSS, a HLA-B44*03^{+} HIV seronegative donor known to respond to the EBV epitope. Cells obtained from this subject were stimulated with a range of concentrations of both the EBV and HIV peptides, and the frequency of cells stimulated to produce IFN-γ was assessed by ELISPOT assay. The results in Figure 4.12 show that the subject's cells made a dose-dependent response to the EBV peptide, but no response to the HIV epitope was seen at even the highest concentration of peptide used to stimulate the cells. Attempts were also made

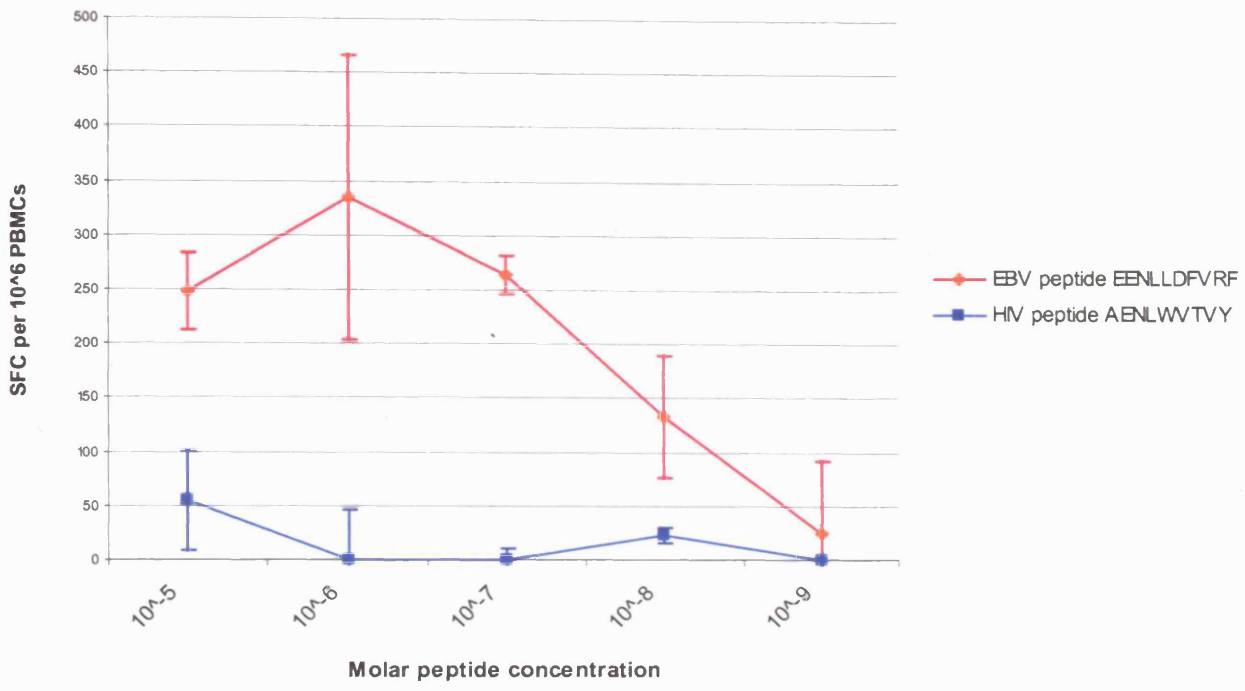


Figure 4.12. Analysis of the response of EBV$^+$, HIV$^-$ subject JSS to the EBV epitope peptide EENLLDFVRF and the HIV epitope peptide AENLWVTVY. PBMCs cryopreserved from subject JSS were stimulated for 24 hours with synthetic peptides representing B44-restricted EBV and HIV epitopes at concentrations between 10^{-5}M and 10^{-9}M. The number of cells stimulated to produce IFN-γ was quantified by ELISPOT assay and is expressed as the mean (of values from duplicate wells) number of specific spot forming cells (SFC) per 10^6 PBMCs (i.e. background values from medium only control wells have been subtracted). The error bars indicate one S.D. above and below the mean.

to expand out a population of HIV-specific $CD8^+$ T cells by *in vitro* culture of PBMCs from subject JSS with the HIV peptide, but this was not successful. Thus there was no evidence of the EBV-specific cells being able to cross-recognise the HIV epitope.

It remained possible that the HIV AENLWVTVY(Y) response had been expanded from memory cells responding to another HLA-B44*03-restricted epitope. To investigate this possibility, the predicted amino acid sequences of the entire proteome of eight common human viruses (influenza virus, EBV, adenovirus, CMV, measles, mumps, VZV and HSV) were screened by Darren Flower (Bioinformatics group, EJIVR, Compton) to identify peptides 9 amino acids in length that conformed to the HLA-B44*03 HLA binding motif (glutamate at position 2 and a hydrophobic residue at the C-terminus (http://www.syfpeithi.de/)), and had chemical similarity to the residues in the HIV AENLWVTVY(Y) epitope, that may have triggered a cross-reactive response. The peptides were ordered according to their chemical similarity to the AENLWTVY peptide, and the top 64 of these, which are listed in Figure 4.13(a), were synthesised, grouped into pools of two, and tested for recognition by cryopreserved PBMCs from WEAU in an IFN-γ ELISPOT assay. As shown in Figure 4.13, a strong response was stimulated by the HIV epitope, but the patient's cells did not cross-recognise any of the test peptides.

Observations made in a second HIV-infected individual, BORI, were initially supportive of the idea that a cross-reactive response could help prime a dominant HIV-specific response. This patient made a dominant response to the HLA-A*29 restricted HIV epitope SFEPIPIHY during acute infection (Jones *et al.*, 2004). Using a similar approach as for that used for WEAU and the HIV AENLWVTVY(Y) epitope to look for possible sequences in other human viruses which could have primed this response, a panel of peptides were tested for cross-recognition by PBMC from BORI in an IFN-γ ELISPOT assay (listed in Figure 4.14(a)). Strong responses were observed towards two sequences derived from the proteome of HSV-2 (Figure 4.14(b)): RLEARLGHL and HFEPAAEPV. However, when the recognition of the HIV and HSV-2 sequences were compared at lower peptide concentrations (by looking at the ability of polyclonally restimulated patient PBMC to lyse target cells pulsed with a range of dilutions of each peptide in a chromium release assay), it was apparent that the two HSV-2 sequences were recognised only at high peptide concentrations (whereas the functional avidity of the response to the SFEPIPIHY peptide was extremely high (10^{-11}-10^{-12}M) (Figure 4.14(c)).

A.

Peptide Pool	Peptide A sequence	Peptide B sequence	Peptide Pool	Peptide A sequence	Peptide B sequence
P1	EEDLRVFGE	EEDLRVFGE	P17	TELLWHAHP	PEDGWVKVH
P2	RELWWVFYA	GENLVTAVS	P18	DENLGVLDR	EENLEGVRR
P3	LEDLSHSVF	TENLMHCVK	P19	DENFYLLVT	PENIWVPVV
P4	AEDLRVLEV	SENLMWDIL	P20	RENLERRGA	TENLGGLSG
P5	YENLVYLYL	ELRSRYWAL	P21	KENLRRPVV	AEQYWRRVV
P6	GEISPLPSL	KEHVIQNAF	P22	NENPEKDVL	CEDLEGRVS
P7	NEGCGWAGW	VEITPYKPTW	P23	EEELWEAVF	PENLLIIIP
P8	FEDLRVLSF	EGGVGWRHW	P24	FENLIPPEI	YENLLRELY
P9	LENLLSRYE	AENLRGLNE	P25	REDPWLDGA	REILWAAYA
P10	MENLRRVYK	AENLISNGI	P26	GENLYVHAD	NEDLYSDMM
P11	LENLRRVLD	TENLRNALQ	P27	NENPYHARR	IESLWVGVY
P12	AENLLLVAL	RENLLTLGQ	P28	PEQPWIAAV	AENLKGVWP
P13	PENPGSRAY	RENLLSGDT	P29	EEIPWVRVY	EELLWFIRG
P14	QENLIEALA	LENLSITEL	P30	AENLPGLLF	GENLKTLRL
P15	WENLLSKAK	AENLSDVTQ	P31	AENLPGLLV	TENLPELWT
P16	YELLYFLVL	KECLWVLSD	P32	LENLKLKLG	EENYYVTVL

B.

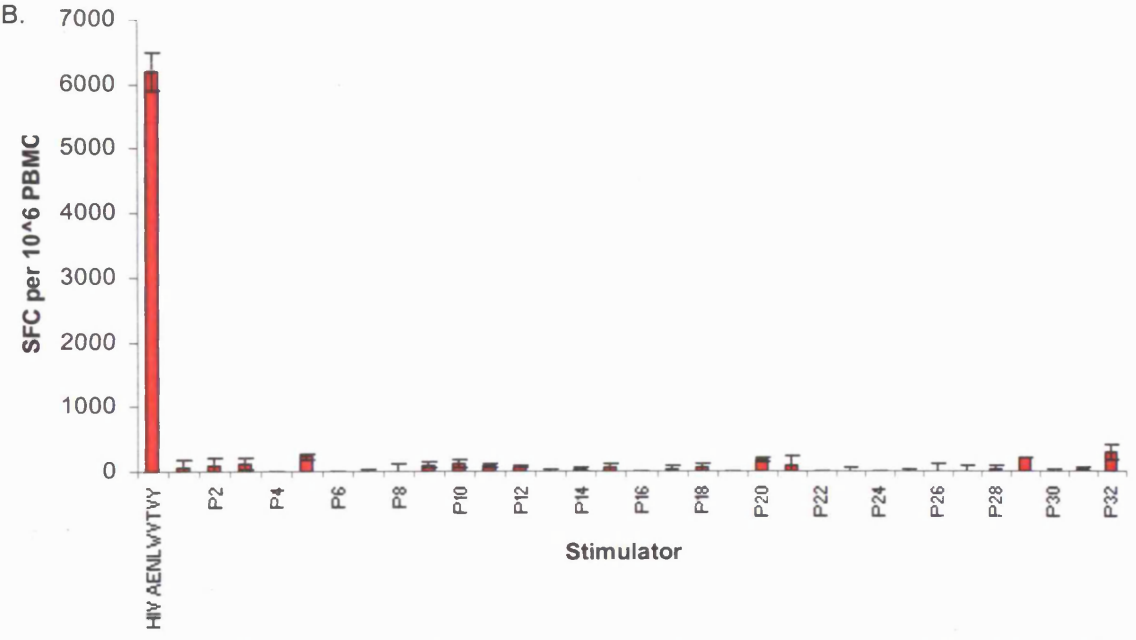


Figure 4.13. Testing peptides representing putative HLA-B44*03-restricted epitopes in unrelated viruses for recognition by PBMCs from patient WEAU using an IFN-γ ELISPOT assay.

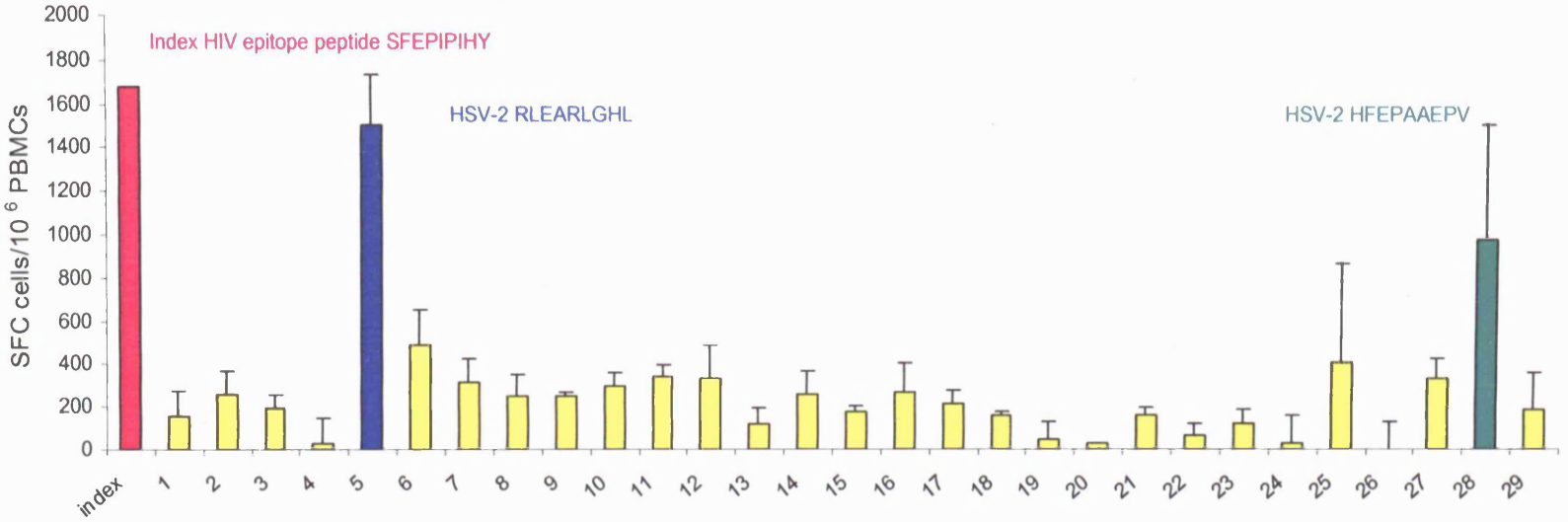
A. The predicted amino acid sequences of the entire proteome of eight common human viruses (EBV, CMV, VZV, HSV, measles, mumps, influenza and adenovirus) were screened by Dr Darren Flower (Bioinformatics Group, EJIVR) to identify peptides 9 amino acids in length that were predicted to bind to HLA- B44*03. These were ranked on the basis of chemical similarity of their constituent amino acids to the HIV epitope AENLWVTVY, and the top 64 were synthesized and combined into pools of two for screening. The amino acid sequences of the peptides in each pool are shown.

B. PBMCs cryopreserved from patient WEAU (20 DFOSx) were stimulated for 24 hours with the indicated synthetic peptides (each peptide at $3x10^{-5}$M): either the immunodominant HIV AENLWVTVY epitope peptide or the peptide pools detailed in (A). The number of cells stimulated to produce IFN-γ was quantified by ELISPOT assay and is expressed as the mean (of values from duplicate wells) number of specific spot forming cells (SFC) per 10^6 PBMCs (i.e. background values from medium only control wells have been subtracted). The error bars indicate one S.D. above and below the mean.

(A)

Peptide	Sequence	Peptide number	Sequence	Peptide number	Sequence
Index	SFEPIPIHY	10	SVEACGNHV	20	NFTPIYIHT
1	TLEPEKNHP	11	GFEAAALHV	21	AFETNQTHD
2	NIEPTYPHG	12	GFEPGTYRL	22	AFEPEVPTP
3	NIEAFLAHL	13	QFEPMLPRV	23	LFNTINFHY
4	DYEPVPRKF	14	GFQPPVTHP	24	NFHPELKKY
5	RLEARLGHL	15	IFETLALRL	25	SFEGDVARV
6	EIEPGVLKV	16	LFHAIPFHM	26	EFEGDFARY
7	PVEPTRPHV	17	RFNPPKMHP	27	RFTPRPQHD
8	DIEGHASHY	18	VFDPAQIHT	28	HFEPAAEPV
9	EYEPEDGEY	19	YFQAHQMHL	29	LFEHLAAHG

(B)



(C)

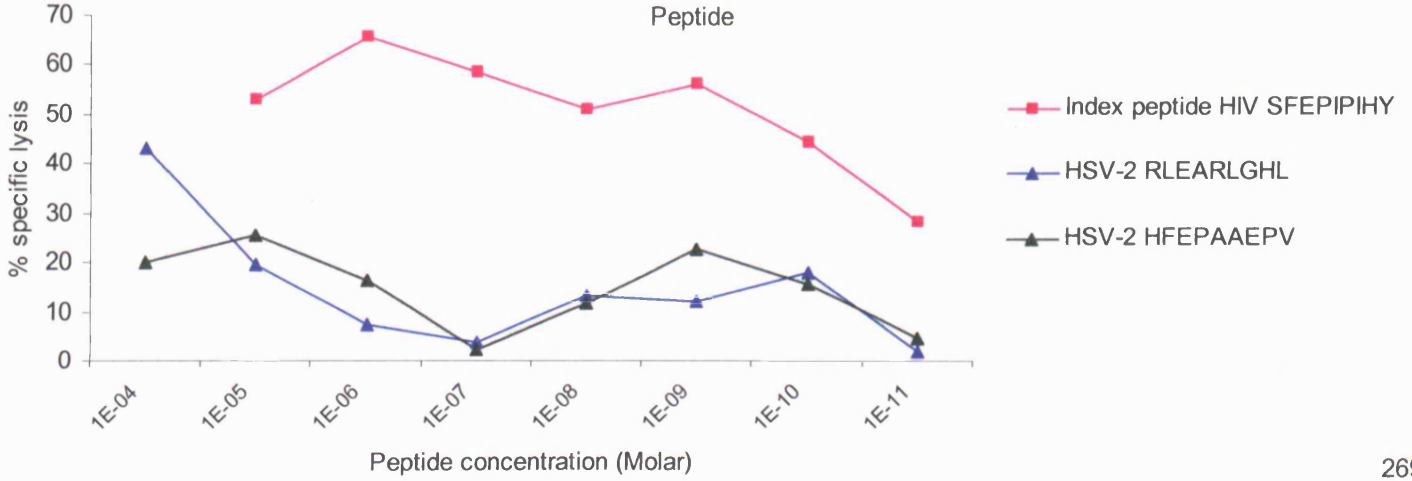


Figure 4.14. Testing peptides representing putative A29-restricted 'SFEPIPIHY-like' viral epitopes for recognition by PBMCs from patient BORI.

(A) Putative A29-restricted viral epitopes which resembled the HIV SFEPIPIHY epitope were predicted in a similar manner as described for the prediction of putative B44-restricted viral epitopes resembling HIV AENLWVTVY in Figure 4.13. The top 29 of these (based upon chemical similarity of their constituent amino acids to the HIV epitope sequence) are listed here.

(B) Synthetic peptides corresponding to the 29 predicted sequences in (A) were synthesized and screened for recognition (along with the immunodominant HIV SFEPIPIHY epitope peptide for comparison) by PBMCs cryopreserved from patient BORI (28 DFOSx). Cells were stimulated for 24 hours with the synthetic peptides (each peptide at 10^{-5}M), and the number of cells stimulated to produce IFN-γ was quantified by ELISPOT assay. The results shown are expressed as the mean (of values from duplicate or triplicate wells) number of specific spot forming cells (SFC) per 10^6 PBMCs (i.e. background values from medium only control wells have been subtracted). The error bars indicate one S.D. above the mean.

(C) The relative recognition of the HIV SFEPIPIHY epitope and HSV-2 derived RLEARLGHL and HFEPAAEPV sequences by PBMCs from patient BORI were determined by ^{51}Cr release assay. PBMC cryopreserved from patient BORI (21 DFOSx) were polyclonally restimulated *in vitro* for 8 days with an anti-CD3 antibody and irradiated allogeneic feeder cells. Their ability to lyse autologous B-LCL target cells pulsed with 10 fold dilutions of the peptides of interest was determined using a standard ^{51}Cr release assay. The results shown are the % specific ^{51}Cr release from the target cells at an E:T of 50:1

This suggested that it was more likely that the HIV-specific CD8 cells were cross recognising the HSV-2 sequences, rather than that HSV-2-specific memory cells had expanded to generate the response to the HIV epitope.

A further question of interest was how commonly cross-reactive responses to HIV-1 epitope peptides are observed in HIV seronegative donors. To gain some insight into this, PBMCs from 10 different HIV seronegative blood donors were stimulated with pools of overlapping peptides that spanned the HIV Gag p24 sequence (listed in Table 4.3) and IFN-γ ELISPOT assays were used to detect responses to the peptides. As shown in Figure 4.15, none of the donors tested made responses to these peptides above the background level of IFN-γ production (that produced by cells incubated with medium alone). To enable more definitive conclusions to be drawn about cross-recognition of HIV sequences by T cells in HIV seronegative individuals, similar studies should ideally have been extended to a large proportion of the HIV-1 proteome and a larger number of subjects, but unfortunately, time and resources did not permit this.

In summary, experiments to identify examples of cross-recognition of HIV epitopes by pre-existing memory T cells of other specificities proved unsuccessful. However it still remained possible that cross-reactivity between HIV and other viral epitopes may influence the nature of the primary HIV-specific $CD8^+$ T cell response. This hypothesis was explored further using murine models.

4.6 Analysis of the relative dependence of naïve and memory $CD8^+$ T cells specific for an H-Y epitope on CD4 help for their expansion

The first aim of experiments in murine models was to test whether, as predicted, memory $CD8^+$ T cells were less dependent on CD4 help for their expansion than naïve $CD8^+$ T cells. This was done by looking at responses to male cells expressing the histocompatibility-Y (H-Y) antigen, as it is known that the primary $CD8^+$ T cell response to this antigen is highly dependent on CD4 help (Simpson & Gordon, 1977). Could expansion of a memory H-Y response occur independently of CD4 help (unlike priming of naïve cells to make a H-Y-specific response)?

In this experiment five groups of four female H-2^b mice were used. The first group were primed with male splenocytes i.p. then 4 weeks later boosted with another inoculation of male splenocytes. A second group of mice were treated in a similar manner as for mice in group 1, but were also given an anti-CD4

	Peptide pools	
P1	MLKETINEEAAEWDRLHPVH	AEWDRLHPVHAGPIAPGQMR
P2	AGPIAPGQMREPRGSDIAGT	EPRGSDIAGTTSTLQEQIAW
P3	TSTLQEQIAWMTNNPPIPVG	MTNNPPIPVGEIYKRWIILG
P4	EIYKRWIILGLNKIVRMYSP	LNKIVRMYSPVSILDIRQGP
P5	VSILDIRQGPKEPFRDYVDR	KEPFRDYVDRFYKTLRAEQA
P6	FYKTLRAEQATQEVKNWMTE	TQEVKNWMTETLLVQNANPD
P7	TLLVQNANPDCKTILKALGP	CKTILKALGPGATLEDMMTA
P8	GATLEDMMTACQGVGGPGHK	CQGVGGPGHKARVLAEAMSQ
P9	ARVLAEAMSQVTNTATMMMQ	VTNTATMMMQRGNFRSPRKT
P10	RGNFRSPRKTIKCFNCGKEG	IKCFNCGKEGHIARNCRAPR
P11	HIARNCRAPRKKGCWKCGQE	KKGCWKCGQEGHQMKDCTER
P12	GHQMKDCTERQANFLGKIWS	QANFLGKIWSSQKGRPGNFP
P13	SQKGRPGNFPQSRLEPTAPP	QSRLEPTAPPEESFRFREET
P14	EESFRFREETTTPSQKQEPI	TTPSQKQEPIDKELYPLTSL
P15	DKELYPLTSLKSLFGNDPSS	KELYPLTSLKSLFGNDPSSQ

Table 4.3. Sequences of overlapping synthetic peptides spanning the HIV-1 Gag p24 sequence. Peptides spanning HIV-1 Gag (based upon the clade B consensus sequence) were used in pools of two in IFN-γ ELISPOT assays (described in Figure 4.15) at a final concentration of 10^{-5}M each.

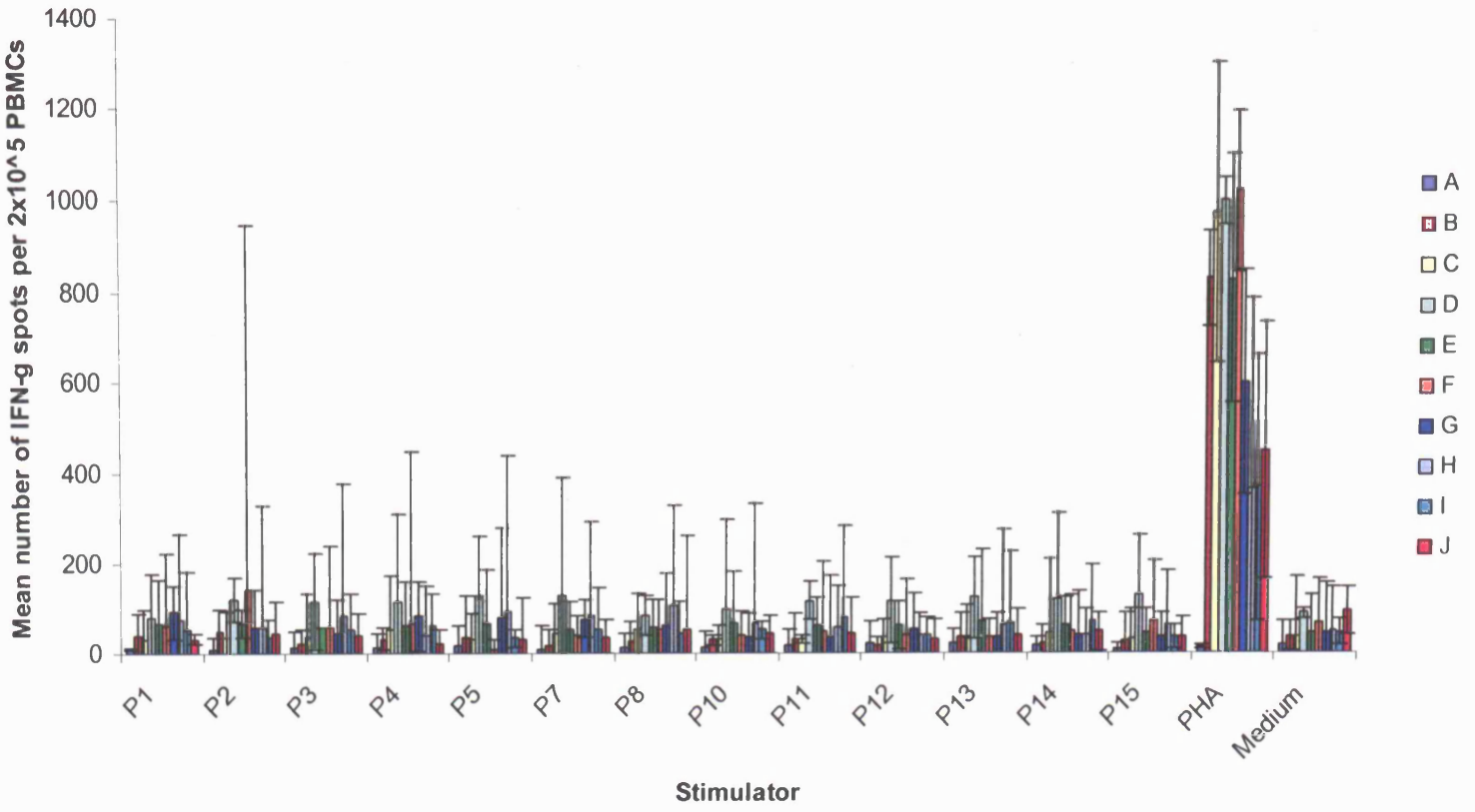


Figure 4.15. Screening PBMCs from HIV-seronegative donors for responses to overlapping synthetic peptides corresponding to the HIV-1 Gag p24 sequence. PBMCs cryopreserved from 10 HIV-seronegative donors (A-J) were incubated for 24 hours with pools of overlapping peptides corresponding to the HIV-1 clade B consensus sequence Gag p24 sequence (Table 4.3) (each at a final concentration of 10^{-5}M) or PHA or medium alone. The number of cells stimulated to produce IFNγ was quantified by ELISPOT assay and is expressed as the mean (of triplicate test wells) number of spots per test well containing $2x10^5$ PBMCs. The error bars indicate one S.D. above and below the mean. N.B. Peptide pools 6 & 9 were in short supply and not included in this experiment.

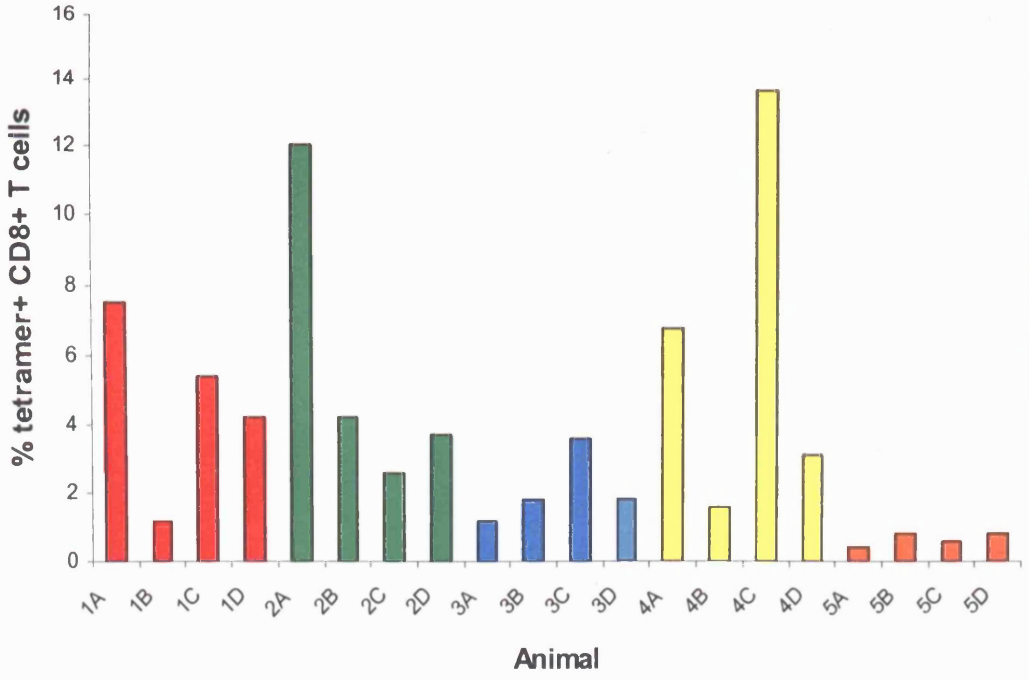
antibody around the time of the boost, so that help was limiting as the memory cells were expanding in response to the boost. In order to check the memory level of H-Y specific cells at ~6 weeks post-priming, mice in a third group only received the initial prime. The fourth group of mice were not initially primed, but were given male splenocytes at the same time as when groups 1 and 2 were boosted, to look at the primary H-Y specific response in intact mice. Mice in group 5 were treated as mice in group 4, but also $CD4^+$ cell-depleted using an anti-CD4 antibody so that CD4 help was limiting as the primary response was being induced. Spleens were taken from mice in all groups on day 13 (post-boost) and H-Y-specific $CD8^+$ T cell responses were assessed for each individual animal by tetramer staining using a H-Y-specific tetramer (D^b/WMHHNMLDI), the results of which are shown in Figure 4.16.

The mean level of tetramer positive cells of mice in groups 4 (primary H-Y-specific response) and 5 (help absent at the time of induction of the primary H-Y-specific response) were 6.3% and 0.7% of $CD8^+$ cells respectively, confirming that this response is a help-dependent one. The mean level of the memory response at 6 weeks post-priming in non-boosted mice (group 3) was found to be 2.1%. In comparison, the response in CD4-depleted and boosted mice (group 2) was higher (5.7%) and was similar to that in non-CD4-depleted boosted mice (group 1 – 4.6%), suggesting that memory cells had expanded efficiently. However, because CD4 T cells were not depleted altogether by the antibody administered, the data do not prove that memory cells can expand in the total absence of help. However, the results obtained here do support the idea that under conditions where CD4 help is limiting, the expansion of a large population of antigen-specific cells from a pre-primed repertoire (group 2) can occur, unlike when starting from a naïve repertoire (group 5), where a similar large expansion was not achieved.

4.7 Investigation of the consequences of the presence of Pichinde virus-primed, LCMV-cross reactive memory cells on the $CD8^+$ T cell response made to LCMV under conditions of $CD4^+$ T cell deficiency

To address the idea that immunodominant HIV-specific responses may be the result of expansion of cross-reactive memory cells, we looked to see how a known cross-reactive memory response could influence the epitope hierarchy in an antiviral immune response being induced under conditions of limited $CD4^+$ T cell help. LCMV and Pichinde virus (PV) are two members of the *Arenaviridae* family, but are genetically and antigenically distinct. The primary

(a)



(b)

Group	Mean % tetramer$^+$ CD8$^+$ T cells
1	4.6
2	5.7
3	2.1
4	6.3
5	0.7

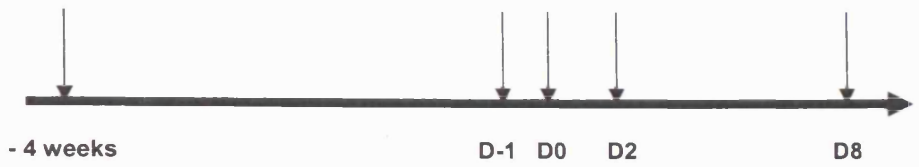
Figure 4.16. Analysis of the relative dependence of naïve and memory CD8$^+$ T cells specific for an H-Y epitope on CD4 help for their expansion. Three groups of female C57BL/6 mice were immunised i.p. with $5x10^6$ splenocytes prepared from male C57BL/6 mice. Groups 1 and 2 were boosted 4 weeks later with a similar inoculation of male splenocytes. Mice in group 3 received no other treatments. Mice in group 2 were also given 1mg anti-CD4 monoclonal antibody GK1.5 i.v. on days –2, 2 and 7 with respect to the day of boosting. A further two groups of mice were not primed initially, but given male splenocytes at the time when groups 1 and 2 were boosted. Mice in group 5 were given a CD4$^+$ cell-depleting antibody as for mice in group 2. The frequency of H-Y-specific cells was determined for all mice on day 13 by staining of B cell-depleted splenocyte suspensions using the D^b/WMHHNMLDI tetramer. Each group consisted of 4 mice (labelled A-D). The percentage of tetramer positive CD8$^+$ cells in (a) individual mice and (b) the mean value for each group are shown above. The efficiency of the CD4 depletion was also checked by staining splenocytes with an anti-CD4 mAb. This showed that the number of splenic CD4$^+$ cells in the depleted mice had been reduced to ~48% of the number seen in the control mice. This experiment was carried out twice, with similar results being obtained each time.

response to each in H-2^b mice is known to only weakly cross-recognise the other virus. However, it has been shown that if mice are already immune to PV the response they make to LCMV then includes a strong PV cross-reactive component due to the outgrowth of cross-reactive clones (Selin *et al.*, 1994). A cross-recognised viral epitope has been identified in the NP205-212 region in both viruses (Brehm *et al.*, 2002). This peptide sequence is highly conserved between LCMV and PV, with variations at only two amino acid residues.

An experiment was carried out to investigate whether mice immune to PV, when CD4 depleted and infected with LCMV, now make an LCMV-specific response that is highly biased towards the PV cross-reactive NP205 epitope (cells responsive to which, being in the memory pool, should be able to expand more efficiently than the naïve LCMV-specific cells). By exploring this, it was hoped to address the question of whether cross-reactive memory T cells have a selective advantage during the establishment of immunodominance when CD4 help is absent.

In Figure 4.17, it can be seen that in this experiment, the primary response to LCMV had the same hierarchy as in the experiment shown earlier in Figure 4.7, with the most dominant responses being towards the GP33 followed by the NP396 epitope, with the NP205 response being the next largest. The mice that had previously been infected with PV and subsequently infected with LCMV, made a slightly lower overall response to LCMV. This could perhaps be due to cross-reactive antibodies in PV-immune mice limiting the amount of LCMV replication and hence antigen. Although the overall response was lower, an enhanced response was made to the NP205 epitope, consistent with previous reports indicating that this could be cross-recognised by PV-specific memory cells. This is reflected by the relative ratios of the magnitude of the response to each epitope in naïve mice and PV-immune mice: for the NP205 epitope, this ratio was greater than one. In primary LCMV infection of CD4-depleted mice, the responses to all epitopes were lower than those in control, undepleted mice, but the epitope hierarchy remained unchanged, as was found in the experiment described in Figure 4.7. In PV-immune, CD4-depleted mice, the response to LCMV reflected that seen in the equivalent non-CD4 depleted group: responses to most epitopes were lower, but that to the cross-recognised NP205 epitope was enhanced. However, the relative ratio of the responses to this epitope in naïve and PV-immune, CD4-depleted mice was very similar to that in the non CD4-depleted groups (1.2 and 1.3 respectively). Thus there was preferential expansion of cross-reactive memory cells when a

(A)



Group 1

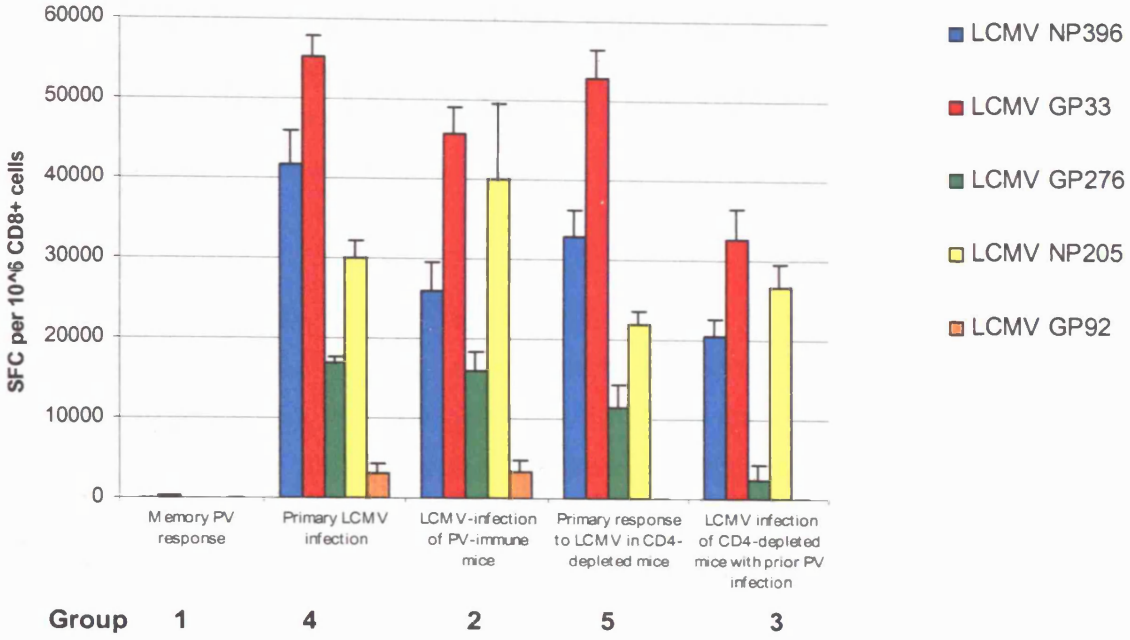
Group 2

Group 3

Group 4

Group 5

(B)



(C)

Epitope	NP396	GP33	GP276	NP205	GP92
PV-cross-reactive?	**No**	**No**	**No**	**Yes**	**No**
Relative magnitude of response to epitope in PV-immune vs naïve mice	0.62	0.83	0.93	1.3	1.0
Relative magnitude of response to epitope in CD4-depleted PV-immune vs CD4-depleted naïve mice	0.62	0.62	0.21	1.2	N.D.*

Figure 4.17. Experiment to address whether cross-reactive T cells in the memory pool become particularly immunodominant in the immune response to LCMV in CD4-depleted mice. As illustrated in (A), three groups of 4 C57BL/6 mice were infected with PV ($2x10^5$ pfu i.p.). Four weeks later, groups 2 and 3 received $2x10^5$ pfu LCMV Armstrong i.p., group 3 having received 1mg anti-CD4 monoclonal antibody GK1.5 on one day prior to and then 2 days after LCMV infection. An additional two groups of mice were infected with LCMV Armstrong only (2×10^5 pfu i.p) at this time, one of the two groups having received 1mg anti-CD4 monoclonal antibody GK1.5 one day prior to and then 2 days after LCMV infection. Administration of the antibody resulted in a reduction in the number of splenic $CD4^+$ cells to <0.2% of the number found in control mice. Eight days after the last LCMV challenge, all groups of mice were culled and splenocytes were incubated with synthetic peptides (at a final concentration of 10^{-6}M) corresponding to LCMV epitopes. The number of cells stimulated to produce IFN-γ was enumerated by ELISPOT assay and was expressed as the mean (of duplicate test wells) number of specific spot forming cells (SFC) per 10^6 $CD8^+$ T cells (i.e. background values from medium only control wells have been subtracted). The results shown in (B) are the mean responses made to each LCMV peptide by groups of four mice, +1SE. The table in (C) shows the relative magnitude of responses to each LCMV epitope in mice with and without prior immunity to PV, i.e. the mean magnitude of the response in PV-immune mice / the mean magnitude of the response in naive mice. These data are representative of results obtained in three independent experiments.

* Not detected

CD8 response was induced to a heterologous virus, but this was not enhanced when CD4 help was absent. One drawback to this experiment was that it has the same problem as that experienced earlier using LCMV infection, in that the virus inherently induces extensive APC activation. Had time permitted, this experiment could have been repeated using recombinant vaccinia viruses encoding the LCMV NP and GP sequences, to try and make the response more help-dependent.

4.8 Discussion

Evidence suggests that in primary HIV infection, there may be preferential expansion of $CD8^+$ T cells to a limited number of immunodominant viral epitopes, especially in those patients who do not control early virus replication well. Pantaleo *et al* showed that there was a difference in the nature of the CD8 response expanded in different patients during acute HIV infection: monoclonal/oligoclonal TCR Vβ family expansions were observed during acute infection in patients who subsequently controlled viral replication poorly, whereas polyclonal TCR Vβ family expansions were observed in those who controlled virus replication well (Pantaleo *et al.*, 1997b). There is also evidence to suggest that there may be differences in the breadth of the HIV-specific $CD8^+$ T cell response expanded during acute infection in patients who control early viral replication with differing efficiency at the epitope level, with those exhibiting poor viral control mounting responses of limited epitope breadth compared to those with good viral control (Jones *et al.*, 2004). In chapter 3, I also found that the primary HIV-specific $CD8^+$ T cell response in patients with moderate-high persisting viral loads tended to be biased towards a limited number of highly immunodominant epitopes.

The work in this chapter focused on investigation of why the primary HIV-specific $CD8^+$ T cell response in patients who establish moderate or high persisting viral loads may be so biased towards a limited number of immunodominant epitopes, exploring mechanisms that may potentially contribute to the preferential expansion of particular epitope-specific T cell responses.

As mentioned in section 4.1, a number of factors influence the epitopes against which $CD8^+$ T cell responses are induced in different individuals and their hierarchy of immunodominance. These include different the HLA alleles an individual possesses (which in turn dictate the specific epitopes and

diversity of those epitopes which are able to be presented to T cells), the efficiency of epitope processing and affinity of epitope binding to MHC, the number and stimulatory capacity of APCs and the repertoire of responding T cells.

The HLA alleles an individual possesses play a key role in dictating which peptides (and the total number of peptides) can potentially be presented to T cells. HLA alleles vary in the diversity of peptides they can present, e.g. HLA-B alleles bind a greater diversity of peptides than HLA-A alleles (Marsh *et al.*, 2000); and a recent study of $CD8^+$ T cell responses in a cohort of HIV-infected individuals from southern Africa found that a greater number of responses were HLA-B restricted than HLA-A restricted (Kiepiela *et al.*, 2004). The particular HLA alleles expressed by a given individual, and the extent of heterozygosity at HLA loci may thus be one of the factors that dictates the epitope breadth and relative immunodominance of responses within the primary HIV-specific $CD8^+$ T cell response. Importantly, there is good evidence to show that the HLA type of an individual has an important impact on the outcome of HIV infection. Certain alleles are associated with good (HLA-B57 (Gillespie *et al.*, 2002; Goulder *et al.*, 1996; Migueles *et al.*, 2000; Tang *et al.*, 2002) and HLA-B27 (den Uyl *et al.*, 2004; Goulder *et al.*, 2001b; Goulder *et al.*, 1997a; McNeil *et al.*, 1996; O'Brien *et al.*, 2001)) or poor (HLA-B35-02/03 (Gao *et al.*, 2001; Jin *et al.*, 2002), HLA-B8 (Candore *et al.*, 1998; Kaslow *et al.*, 1990; McNeil *et al.*, 1996)) ability to restrict viral replication and disease prognosis. Also, HLA heterozygosity in HIV-1 infected individuals is advantageous, with those heterozygous at HLA class I A, B and C loci having a better disease prognosis than those individuals who are homozygous for one or more loci, who progress to AIDS more rapidly (Carrington *et al.*, 1999). There are a number of possible explanations for these associations. The 'good' alleles for example may present immunodominant epitopes which are associated with responses of particularly high efficacy: the responses may target epitopes within proteins that are expressed early in the viral life cycle; or the responses involved may be those for which viral escape is restricted by high costs to viral fitness, as has been suggested in the case of HLA-B57- and HLA-B27-restricted epitopes (Goulder & Watkins, 2004). It is also possible that the T cells involved in the response to certain peptide-HLA combinations may use 'better' TCRs, which recognise the epitopes with high avidity and/or have high functional flexibility.

However these associations may also relate in part to the number of epitopes that the HLA alleles are able to present (Nelson *et al.*, 1997). Broader HIV-specific responses have been shown to be induced in the context of the 'good' alleles, with responses induced to more viral epitopes (perhaps more T cell clones are involved in the response to each epitope too). This evidence would fit with the idea that presentation of a large number of epitopes (as would be allowed for by HLA heterozygosity) is beneficial. Based on these observations, it is therefore possible that the biasing of the response towards a limited number of dominant epitopes in patients who established high persisting viral loads may be explained at least in part, by the influence of HLA type. It is notable that 5 out of the 13 patients in the cohort studied here expressed HLA-B8. Since the frequency of this allele reported in Caucasian populations is 10% (HLA 1991), this allele is over-represented in this patient cohort, which may explain the association of this group with a bad prognosis overall.

However, HLA type is not the only factor which could influence the array of epitope peptides bound to MHC class I molecules on the surface of APCs and specificity of the $CD8^+$ T cell response subsequently primed. The relative quantities that are produced of different viral proteins can affect the relative abundance of different peptide-MHC complexes, e.g. Tsomides *et al* found that the abundance of complexes of HLA-A2 with a Gag and a RT-derived epitope was ~400 and ~12 molecules per HIV-infected Jurkat-A2 cell respectively, an observation attributed to the relatively low levels of RT produced in HIV-infected cells (Tsomides *et al.*, 1994). Host antigen processing pathways may also dictate whether particular epitope peptides are generated during an infection, e.g. in mice infected with AKV/MCF type murine leukaemia virus (MLV), an epitope in the p15E transmembrane viral envelope protein constitutes the immunodominant sequence recognised by virus-specific CTL; however the corresponding sequence in Friend/Moloney/Rauscher type MLV, which differs by just a single amino acid residue, does not induce a CTL response, because the amino acid difference causes epitope destruction by specific proteasomal cleavage (Ossendorp *et al.*, 1996). In addition, the affinity of binding of epitope peptides to their restricting MHC alleles influences their presentation. Peptides that have a high MHC binding affinity are likely to compete efficiently with both other viral peptides and host peptides for presentation and be represented at relatively high levels on the host cell surface. Peptides with extremely high MHC binding

affinities have been shown to block the expansion of T cell responses to other epitopes presented by the same MHC molecule both *in vitro* and *in vivo* (von Herrath *et al.*, 1998).

In addition to the factors discussed above, which all affect the array of epitope peptides presented to $CD8^+$ T cells, the epitope specificity and immunodominance hierarchy of a virus-specific $CD8^+$ T cell response is also affected by the repertoire of host T cells available to participate in the response. The host T cell repertoire is initially shaped by positive and negative selection in the thymus and is subsequently modified by selective forces in the periphery. Negative selection may result in no response (or only a weak response) being mounted to certain viral epitopes that resemble epitopes derived from self proteins, as demonstrated using transgenic models (von Herrath *et al.*, 1994). The host T cell repertoire can be selected for during an infection, and then be further modulated as a result of encounter with the same or new pathogens (Lin *et al.*, 2000). For example, acute LCMV infection elicits a virus-specific $CD8^+$ T cell response which substantially alters the host's T cell repertoire (Butz & Bevan, 1998; Murali-Krishna *et al.*, 1998; Selin *et al.*, 1999; Welsh *et al.*, 1997); this repertoire is preserved into the memory state such that it remains skewed after the virus has been cleared (Murali-Krishna *et al.*, 1998; Selin *et al.*, 1999). Further selection of the memory T cell repertoire can occur upon secondary infection with a homologous virus. This may involve affinity maturation (Busch & Pamer, 1999; Busch *et al.*, 1998) and changes in the diversity of the secondary T cell repertoire (Busch *et al.*, 1998; Maryanski *et al.*, 1996). The existing T cell repertoire can also be perturbed by encounter with heterologous viruses which can quantitatively reduce and qualitatively alter the $CD8^+$ T cell memory to previously encountered pathogens. Loss of memory T cells specific for previously encountered pathogens may occur following subsequent viral infections due to competition for space in the memory pool to accommodate the entry of new T cell populations (Selin *et al.*, 1996; Selin *et al.*, 1999). Subsequent heterologous viral infections may also result in alteration of the hierarchy of a T cell repertoire, as both distantly related and unrelated viruses are able to reactivate virus-specific memory T cells through cross-reactivity, as shown by LCMV, PV, VV and MCMV infection of mice (Brehm *et al.*, 2002; Selin *et al.*, 1996; Selin *et al.*, 1999).

Because cells in the memory pool are present at higher frequencies and are also more readily triggered to expand on antigenic exposure, this allows them to mount accelerated responses to recognised/cross-recognised pathogens. This not only has a positive effect on the response to an epitope that is recognised/cross-recognised by cells in the memory pool but can also have a negative effect on responses to other viral epitopes, for example IFN-γ produced by the rapidly responding cells can suppress expansion of responses of other specificities (Rodriguez *et al.*, 2002).

The number and stimulatory capacity of the APCs present at the time a response is primed may also have an important impact on the breadth of epitopes to which responses are induced and their relative immunodominance. During primary HIV infection APC function may be impaired. Possible reasons for this may be direct or indirect effects of the virus on APC numbers or activity, or effects secondary to lack of CD4 help for the activation of APCs. This may result in defects in antigen capture, processing and presentation, and T cell activation (reviewed in (Loré & Larsson, 2003)). Under conditions where $CD4^+$ T cell help and the number and/or functional capacity of APCs are limited the epitope and clonal breadth of the $CD8^+$ T cell response induced may be restricted, with a tendency for responses that require the least help/co-stimulation to be particularly competitive and hence to be preferentially expanded. As discussed below, these may include responses to high affinity epitopes and/or responses involving high avidity T cell-APC interactions; and responses mediated by T cells in the memory pool. The first issue explored in this chapter was whether high avidity responses were particularly dominant during the primary HIV-specific $CD8^+$ T cell response.

A study by Franco *et al* suggested that the binding affinity of a peptide epitope for its class I restriction element is a key factor in determining helper independence (Franco *et al.*, 2000). Immunogens that bound MHC class I with high affinity were found not to require help for priming of antigen-specific CTL, but for those with intermediate MHC binding affinity a source of help (provided by either a class II MHC-restricted T helper cell epitope or administration of antibody to CD40) was required to obtain significant CTL priming. A possible explanation for this association is that the concentration of an epitope on the surface of an APC and/or length of time that the epitope is expressed above

some threshold concentration (both of which may be increased if an epitope interacts with MHC with high affinity) may be critical factors in determining need for help. If a sufficiently strong and/or sustained antigenic signal is provided, then help may not be required. In addition to this, the TCR affinity for the peptide-MHC complex is likely to be important too.

I chose to measure the functional avidity of epitope-specific $CD8^+$ T cell responses (as this would give insight into the combined effects of peptide affinity and TCR affinity), and to explore how this was related to their immunodominance in the primary HIV-specific $CD8^+$ T cell response. For this analysis, both the functional avidity and the magnitude of epitope-specific $CD8^+$ T cell responses should have been measured at timepoints in acute/subacute infection, and the relationship between functional avidity and relative immunodominance calculated from this data. However, due to limitations on sample availability, studies were instead carried out using cells cryopreserved at timepoints in early infection. Data presented in Jones *et al* (Jones *et al.*, 2004) indicate that by early infection, the HIV-specific $CD8^+$ T cell response typically becomes less biased towards the epitopes that were highly immunodominant in acute/subacute infection, with subdominant responses becoming more apparent, but that dominant responses are still observed to those epitopes that stimulated the strongest responses in acute infection. Analysis of the functional avidity of responses that were dominant at this time should thus provide a reasonable picture of those responses that were immunodominant in acute/subacute infection.

Efforts were made to measure the functional avidity of epitope-specific responses (defined as the concentration of peptide required to stimulate half a maximal response when titrated concentrations of epitope peptide were used to stimulate patient PBMC in IFN-γ ELISPOT assays) using cells cryopreserved as early as possible in infection (preferably at the same timepoints at which the relative magnitude of different epitope-specific responses was analysed in chapter 3). However in some cases it was necessary to use cells from slightly later timepoints for this analysis. As different clones of T cells may be used in an epitope-specific response over the course of infection, there is potential for the avidity of the response to change (how the avidity of a T cell response may alter during the course of infection is explored in chapter 5), and so measurements of functional avidity made at a later timepoint may not reflect that at earlier timepoints.

Despite drawbacks to this analysis, this analysis revealed there was a trend towards an association between the functional avidity and relative dominance of epitope-specific responses in early HIV infection, with the more dominant T cell responses tending to display higher functional avidities than less dominant responses. Also, when the functional avidities of the dominant responses in patients who subsequently established different persisting viral loads were compared, the responses of the highest viral load group appeared to display the highest functional avidities (although this part of the analysis was limited by the small number of patients studied). It would also have been of greater interest to address the functional avidity of dominant $CD8^+$ T cell responses in patients who established very low persisting viral loads (in whom help/APC activation may be best preserved), but unfortunately no such patients were available for study. These results suggest that in this cohort of HIV-infected individuals there is a relationship (although not absolute) between immunodominance and avidity; and more importantly provide suggestive evidence for dominant responses being of particularly high functional avidity in patients who established high persisting viral loads (where APC functions may be most compromised). Interestingly, Cao *et al* (Cao *et al.*, 2003) did not observe a relationship between the avidity of epitope-specific responses and the overall frequency of the cells recognising the epitope in patients studied during primary HIV-1 infection. The reason(s) for this are not clear, but could relate to the fact that this study addressed the relationship between the avidity and the absolute magnitude (rather than the relative dominance) of responses, and as different subjects were studied during acute, subacute or early infection (a time-frame over which response dynamics alter rapidly), no overall relationship between response magnitude and functional avidity was apparent. Is there a relationship between immunodominance and functional avidity in $CD8^+$ T cell responses to other viruses? Results from analysis of the antiviral CTL response elicited in BALB/c mice following i.n. infection with Simian Virus 5 (SV5) suggested that there is not a positive correlation between avidity and immunodominance in this system. Analysis of the SV5-specific CTL response identified an immunodominant response to be directed against an epitope within the M protein and subdominant responses to be directed against epitopes in the P, F and HN proteins of the virus (Gray *et al.*, 2001a). The avidities of these responses were subsequently assessed using a modified ELISPOT assay to measure the contribution made by low avidity CTL to each protein-specific response. The determination of the relative proportion of high

and low avidity CTL 6 days after immunisation revealed that while the immunodominant M protein-specific response and subdominant F- and HN-protein specific responses were comprised of both high and low avidity CTL, the subdominant response directed against the epitope present in the P protein was exclusively high avidity. From these results, it was hypothesised that the size of an immunodominant response could be due in part to activation of lower-avidity precursors in addition to higher avidity precursors. By contrast, the size of a subdominant response could be the result of elicitation of only a subset of CTL which possess a restricted avidity. The method used for analysis of the avidity of HIV-specific responses studied here did not allow for the differentiation of high and low avidity T cells within individual immunodominant and subdominant responses.

By contrast, investigation of T cell responses made to a panel of minor histocompatibility antigens in a murine model showed functional avidity to partially reflect the hierarchy of the response (Yoshimura *et al.*, 2004), and assessment of LCMV-specific responses also provide some support for a relationship between the avidity of a response and its relative dominance. As can be seen form the data summarised in Table 4.1, the NP396-specific epitope elicits the highest magnitude response in LCMV-infected C57BL/6 mice, and this is also the highest avidity response too, being able to sensitise target cells for CTL lysis at very low peptide concentrations.

In summary, this part of the study provided some support for there being preferential expansion of responses of high functional avidity during the primary HIV-specific $CD8^+$ T cell response, but did not provide definitive evidence to indicate that this was particularly favoured in the context of limited help/APC activation. This was addressed more directly in mouse models.

The $CD8^+$ T cell response made to LCMV in $H\text{-}2^b$ mice was studied initially as viral epitopes recognised in this mouse strain are well characterised (Gairin *et al.*, 1995; Klavinskis *et al.*, 1990; Oldstone *et al.*, 1988; Schulz *et al.*, 1989; van der Most *et al.*, 1998) and the immunodominance hierarchy of the response has been addressed in previous studies (Murali-Krishna *et al.*, 1998; van der Most *et al.*, 2003; van der Most *et al.*, 1998). Help for the priming of the LCMV-specific CD8 response was removed by administration of a $CD4^+$ cell depleting antibody (or in later experiments using IA k/o mice) and the response made to LCMV in CD4 cell deficient mice vs. control mice was assessed by looking at the relative hierarchy of responses made to epitope

peptides in an IFN-γ ELISPOT assay. Results from this experiment showed that the LCMV-specific $CD8^+$ T cell response induced in CD4-depleted mice was of slightly lower magnitude than that induced in control mice, but the hierarchy of immunodominance of responses to individual viral epitopes was not markedly altered.

Interestingly, the hierarchy of responses observed in control mice was not in agreement with other published studies. First I found that the response to the GP33 epitope was of higher magnitude than that to the NP396 epitope. The data in the literature regarding the relative immunodominance of responses to the NP396 and GP33 epitopes is controversial, with some studies reporting that NP396 is more dominant than GP33 (Murali-Krishna *et al.*, 1998; Wherry *et al.*, 2003a), and others finding that the opposite is true (Gallimore *et al.*, 1998). In the IFN-γ ELISPOT assays I carried out, it was always seen that GP33 elicited a higher magnitude response than NP396. However, when tetramers were used to read out the response to both D^b-restricted epitopes, the NP396 response was more dominant (data not shown). This discrepancy may be due to some of the cells responding to the high affinity NP396 epitope being 'exhausted' and being unable to make IFN-γ in the ELISPOT assay – the response to this epitope (but not the GP33 epitope) exhausts readily following high dose LCMV infection (Zajac *et al.*, 1998). Alternatively it could be explained by the fact that the sequence of the D^b restricted GP33-41 epitope partially overlaps with another epitope, GP34-41, which is presented in the context of K^b. Therefore functional analysis of GP33-41 specific cells is complicated by the simultaneous presentation of the GP34-41 peptide accounting for a proportion of the IFN-γ response seen to be elicited by the GP33-41 peptide. Tetramer staining only detects cells in a MHC-restricted manner, so a D^b tetramer would only specifically detect the D^b-restricted GP33-specific cells. I also observed a larger response to the NP205 epitope than would be expected on the basis of published data. In early experiments I carried out (data from which is not shown) the response to NP205 was in agreement with the published hierarchy, with it being a minor epitope inducing a response somewhere between the level induced by GP276 and GP92. However, in later assays using the same peptides, the NP205 response emerged as a more dominant response. Whether this had something to do with the peptide being chemically unstable, giving rise to an atypical response, is not clear.

Another aspect of the CD8 response primed in the absence of CD4 help which was explored was the clonality of the epitope-specific response. No major differences, such as a more restricted TCR Vβ family usage, were observed in the utilisation of various TCR Vβ families by LCMV-specific cells in CD4-depleted and control mice.

One drawback of using the LCMV infection model to explore the effect of lack of $CD4^+$ T cell help at the time of priming on the hierarchy of the CD8 response induced is that a robust primary $CD8^+$ T cell response can still be induced to this virus in the absence of help, as observed here and in previous studies (Christensen *et al.*, 1994; Leist *et al.*, 1987; Moskophidis *et al.*, 1987b; Rahemtulla *et al.*, 1991). There is controversy in the literature over the requirement for CD4 help for priming of $CD8^+$ T cell responses. Some studies suggest that it is not needed (Aichele *et al.*, 1990; Buller *et al.*, 1987; Janssen *et al.*, 2003), whilst other suggest that it is required. The requirement for $CD4^+$ T cell help for primary CD8 expansion can be demonstrated most clearly in situations where there is no 'adjuvant' effect present, for example in the response to the H-Y antigen (Millrain *et al.*, 2001) or cross-priming of responses to protein antigens (Bennett *et al.*, 1997). In the 'help-independent' systems, a weight of evidence suggests that the APC activation necessary to see expansion of naïve $CD8^+$ T cells is substituted for by signalling through pattern recognition receptors responsive to pathogen components (e.g. TLRs), and innate/inflammatory cytokines (Hertz *et al.*, 2001; Janeway & Medzhitov, 2002; Le Bon *et al.*, 2003). Hence in an infection such as LCMV where there is a high level of virus replication in lymphoid tissues and a strong innate cytokine response, the primary $CD8^+$ T cell response is expanded almost as well in the absence of help as in its presence. Weaker responses are seen when there is less APC activation by viruses. In studies using two subtypes of influenza virus that differed in their requirement for CD4 help for the CD8 responses they elicited, it was found that the H2N2 subtype (help-independent) induced co-stimulatory activity on APCs, but that the H1N1 subtype (help-dependent) did not (Wu & Liu, 1994). Co-stimulation provided by APCs infected with H2N2, but not H1N1, were able to induce the efficient clonal expansion and functional maturation of $CD8^+$ T cells.

In order to overcome the problem of the CD4-independence of the LCMV-specific CD8 response, I also carried out experiments to look at the effects of CD4 help on the hierarchy of the LCMV-specific CD8 response induced using recombinant vaccinia viruses expressing LCMV epitopes, as it was thought

this infection may induce less co-stimulation. Results obtained using this system did provide some evidence to support the hypothesis that the $CD8^+$ T cell response to the high affinity NP396 epitope was slightly favoured in the absence of $CD4^+$ T cell help, although this was not statistically significant. However it was notable that here, as in LCMV infection, lack of $CD4^+$ T cell help caused only a small reduction in the overall magnitude of the virus-specific $CD8^+$ T cell response, suggesting that here too, sufficient APC activation was occurring to support near-optimal $CD8^+$ T cell expansion.

Another system used was influenza virus infection of mice, as it was predicted the $CD8^+$ T cell response to influenza virus inoculated i.p. would possibly be more help-dependent. This was also another model for which a number of H-2^b-restricted epitopes have been well characterised. However, the information regarding the MHC binding affinities of the influenza peptides in the literature was incomplete, so this was determined experimentally. There were discrepancies between the results of all studies addressing this, both those in the literature and the results obtained here. This could be due in part to differences in the assays used to measure peptide binding affinities. Two types of assay are traditionally used: stabilisation assays (described in section 4.3) and competition assays. In competition assays, increasing concentrations of a competitor test peptide are added to cells which have been radiolabelled with a probe and the concentration of the competitor required to yield 50% inhibition of the binding of the radiolabelled probe is used as a measure of its affinity. Although the two assays give different measures of affinity, a series of peptides tested using both methods should give similar results in terms of the hierarchy the peptides are placed in. However, differences are also apparent between results obtained using the same assay (as can be seen from comparison of the measurements of LCMV peptide MHC binding affinities made by different groups shown in Table 4.1). The reasons for this variation are unclear; discrepancies may potentially relate to differences in the quality of the peptides used in different studies.

Other, more novel methods which provide a more direct measurement of peptide binding to MHC, such as an ELISA method, could potentially have been used to try and obtain a more reliable picture of the MHC binding affinities of the influenza (and LCMV) peptides. In this type of assay, recombinant MHC class I heavy chain molecules are diluted in a buffer containing β_2m and the appropriate peptide, and peptide-MHC complexes fold in a peptide-dependent manner. The complexes can be captured on a plate

coated with a conformation-dependent antibody and subsequently detected by a quantitative ELISA assay. How many complexes are generated can be plotted as a function of how much peptide is offered from the results of the ELISA, and the affinity of the peptide-MHC interaction thereby determined (Sylvester-Hvid *et al.*, 2002).

When the response to influenza was measured in IA k/o mice, there was evidence to suggest that the response to this virus was slightly more help-dependent compared to that to LCMV or VV-LCMV. When the total magnitudes of responses to all epitopes were compared for the control and CD4-deficient mice, that to LCMV was reduced by 19% in the CD4-deficient mice, that to VV-LCMV by 18%, and that to influenza by 34%. However despite this, no evidence was obtained to suggest that there was marked expansion of responses to the highest affinity epitopes when help was limited at the time of induction of the $CD8^+$ T cell response. These findings are in agreement with the results of a study by Chen *et al* (Chen *et al.*, 2002) who examined the influence of CD4 help on the immunodominance hierarchy in C57BL/6 mice infected i.p. with influenza PR8 virus by using mice with targeted deletions of CD4 or the $I\text{-}A^b$ β chain. It was found that absence of CD4 help resulted in a decrease in the number of responding splenic $CD8^+$ T cells but had little effect on the immunodominance hierarchy of the response.

In summary, in none of the viral infections used was any strong evidence seen for there being a preferential expansion of responses to high affinity epitopes or of high avidity responses when CD4 cells were absent. A trend was observed towards lower magnitude responses being made in the absence of CD4 help, but the overall immunodominance hierarchies of epitope-specific CD8 responses were not greatly affected by absence of CD4 cells. It would have been interesting, had time permitted, to repeat the epitope hierarchy experiments using mice that may better mimic the defects in APC numbers/function thought to be present in acute/early HIV-1 infection.

One study has suggested that during primary HIV-1 infection, DCs expressing low levels of co-stimulatory molecules accumulate in lymph nodes (Lore *et al.*, 2002). Reports in the literature suggest that absence of CD28-mediated co-stimulation does not affect the hierarchy of epitope immunodominance of virus-specific $CD8^+$ T cell responses in mice. For example, Christensen *et al* found that when the LCMV-specific response to the two immunodominant epitopes was compared in experiments using $CD28^{-/-}$ mice, there was no evidence for enhanced biasing of the response to the high affinity NP396

epitope in the absence of CD28 signalling (Christensen *et al.*, 2002). Likewise, Chen *et al* who addressed the role co-stimulation plays in establishing epitope immunodominance hierarchies in mice infected with influenza virus found that when B7-mediated signalling was interrupted, epitope-specific $CD8^+$ T cells retained their relative positions in the hierarchy (although it positively influenced the activation of naïve virus-specific CD8 cells of all epitope specificities) (Chen *et al.*, 2002). Interestingly however, other studies have indicated that in the absence of CD28 co-stimulation, CD8 responses were exclusively directed at immunodominant tumour peptides, whereas CD8 responses against subdominant epitopes only occurred when CD28 co-stimulation was present (Johnston *et al.*, 1996). Hence in the virus infection models, infection-associated innate activation may be able to substitute for engineered defects in co-stimulation. Importantly however, during primary HIV-1 infection there are likely to be multiple concurrent immunological abnormalities, in both APC numbers and functional capacity and the availability of CD4 T cell help. Under these conditions, infection-associated APC activation may be insufficient to 'rescue' adequate APC functions. Experiments could potentially be done in murine models to limit APC number and/or function more dramatically (using for example combinations of antibodies to block multiple pathways of help/co-stimulation, chimeric mice deficient in APC subsets, or chronic LCMV infection which reduces APC function) and the effects on the nature of the CD8 response induced following virus infection under these conditions addressed.

Not only can $CD4^+$ T cells play a role in the primary expansion of $CD8^+$ T cells and their differentiation into cytotoxic effector cells, but it has also been shown that secondary CTL expansion is completely dependent on the presence of helper T cells during, but not after, priming (Janssen *et al.*, 2003; Shedlock & Shen, 2003; Sun & Bevan, 2003). In a virus infection, CD4 cells may thus be more important for secondary rather than primary CTL expansion, or in the context of a chronic viral infection such as HIV, for maintaining CD8 T cell responses. This has been demonstrated in LCMV (Matloubian *et al.*, 1994) and MHV-68 infection (Cardin *et al.*, 1996) in mice. In chronic LCMV infection of CD4-depleted mice, the response does not get biased towards NP396 because these cells are deleted by exhaustion (Zajac *et al.*, 1998). A changing pattern of epitope dominance has been observed during acute and persistent murine herpes virus (MHV)-68 infection in intact mice, which is thought to

relate to differential expression of lytic/latent viral proteins during different phases of the infection (Stevenson *et al.*, 1999); but MHV-68 infection (which unlike LCMV, is a lytic virus) of I-Ab-/- mice eventually results in death as a consequence of continuing, low-level infection (Cardin *et al.*, 1996) (thought to be due to the absence of IFN-γ producing $CD4^+$ effector cells which mediate direct control of the infection (Christensen *et al.*, 1999)). These models thus do not provide full insight into how the CD8 T cell repertoire may evolve over time in the absence of CD4 T cell help. During chronic HIV infection limited availability of CD4 help may perhaps lead to biasing of responses to high avidity responses over time. This is addressed in chapter 5. Here, the focus was on what may be happening in primary HIV infection, when $CD8^+$ T cell responses were expanded initially.

Another possibility as to why certain responses might be preferentially able to expand under conditions where APC functions and/or CD4 help are suboptimal (in addition to the idea that they may be specific for epitopes with high affinity for MHC restriction elements or particularly high avidity responses) is if they are mediated by memory cells. Memory T cells possess a number of properties which enable them to respond early upon secondary encounter with a pathogen and be protective. They persist at relatively high frequencies, express high levels of adhesion molecules and, being constitutively at a higher state of activation, are easier to stimulate than naïve cells and can also be triggered by relatively low affinity interactions through their TCRs (Lau *et al.*, 1994; Pihlgren *et al.*, 1996; Razvi *et al.*, 1995; Selin & Welsh, 1997; Selin *et al.*, 1996; Sprent & Tough, 1996; Tabi *et al.*, 1988; Tough & Sprent, 1994; Tough *et al.*, 1996). When a CD8 response is induced to HIV, pre-existing memory cells may be present that were raised to some previous infection, and are able to cross recognise an HIV epitope. These memory cells may have the capacity to expand more rapidly than naïve cells, and this may be accentuated when there is a lack of CD4 help.

One question I addressed, using murine models, was whether as predicted, memory $CD8^+$ T cells are less dependent on CD4 help for their expansion than naïve $CD8^+$ T cells. An experiment was carried out to look at the help-dependency of the secondary response of female mice to the male H-Y antigen, the primary CD8 response to which is known to be dependent on CD4 help (Simpson & Gordon, 1977). The response made to male

splenocytes was assessed in appropriate groups of CD4-depleted mice which had or had not been previously primed (the initial prime occurring in the presence of CD4 help). Tetramer staining of cells revealed that an average of 5.7% of $CD8^+$ cells were H-Y-specific in the pre-primed mice boosted in the absence of CD4 help, whereas the memory level of H-Y-specific cells in unboosted mice was only 2.1%. Expansion of H-Y-specific cells memory cells thus did occur, supporting the idea that expansion of a memory H-Y response can occur independently of help, unlike priming of naive cells to make a H-Y response.

However, this experiment was not ideal in that the antibody-mediated removal of $CD4^+$ cells was not as efficient as was hoped for, and some help was likely present for the expansion of the secondary response. To ensure the total absence of help, an experiment involving the adoptive transfer of pre-sorted tetramer positive memory cells into IA k/o mice and following their expansion upon secondary activation would have been required. Use of IA k/o mice would have also addressed the potential problem that the primed H-Y-specific CD4 cells may have be harder to deplete than the general $CD4^+$ T cell pool due to an altered activation state or *in vivo* location.

As briefly reviewed earlier in the discussion, previous studies in murine models have demonstrated that there can be cross-reactivity in CD8 responses between distantly related viruses (e.g. LCMV and Pichinde virus) or even unrelated viruses (e.g. LCMV and vaccinia virus) (Yang & Welsh, 1986; Yang *et al.*, 1989). It has also been shown that if there are cross-reactive $CD8^+$ T cells in the memory pool, these are preferentially expanded in the immune response to the second virus (Selin *et al.*, 1994), and this can confer cross protection too (Chen *et al.*, 2001; Selin *et al.*, 1998). For example, a study by Brehm *et al* (Brehm *et al.*, 2002) using PV and LCMV infection of C57BL/6 mice showed that previous infection with either virus provided partial protection against infection with the other virus, and that this protection was mediated by the presence of cross-reactive $CD8^+$ T cells in the memory pool. Furthermore, it was also shown that in the $CD8^+$ T cell response elicited by LCMV infection in PV-immune mice, there was a reduced frequency of cells responding to normally immunodominant epitopes and expansion of cross-reactive NP205-specific T cells (which are normally subdominant), i.e. the hierarchy of the LCMV-specific response differed from that normally seen in C57BL/6 mice. Selective expansion of cross-reactive memory cells thus can

occur during an antiviral immune response, and impact on the hierarchy of epitope immunodominance.

In this study, I addressed the question, if help was limiting during the induction of a CD8 response, do cross-reactive memory responses expand to become even more immunodominant than when help is present? To study the effects of the presence of a pre-existing pool of memory cells on an antiviral response induced under conditions where CD4 help was limited, PV immune mice were CD4 depleted and then infected with LCMV. I found that the response to the NP205 epitope was enhanced in LCMV-infected mice with prior immunity to PV compared to naïve LCMV-infected mice, confirming that this was a cross-reactive epitope; but did not observe this effect to be accentuated even more when the response to the second virus was induced in the absence of CD4 help. However given the lack of help-dependency of the LCMV-specific response, as discussed earlier, this result is perhaps not surprising. A similar experiment using recombinant vaccinia viruses encoding LCMV structural genes may experience the same problem, and so future experiments to re-address this question in a situation where the number of activated APCs is limited (approaches to achieving which were discussed above) may be of more value.

Cross-recognition of epitopes from different viruses by human $CD8^+$ T cells has also been reported. For example, an immunodominant HLA-A2 restricted epitope in HCV shares 7 out of 9 amino acids with an immunodominant influenza epitope (Wedemeyer *et al.*, 2001), and T cells have shown to cross-react with the two epitopes. Another example of this is the cross-reactivity found between EBV and influenza (Welsh *et al.*, 2004). Cross-reactive T cells specific for the main HLA-A2 restricted immunodominant peptide of EBV and main HLA-A2 restricted immunodominant peptide of influenza have been described, even though the peptides have only three amino acids in common. How a host responds to an infectious agent could therefore be influenced by its history of previous infections and their influence on the memory T cell pool. Is it possible that a history of influenza infection might confer a level of resistance to HCV? Does a strong presence of influenza-induced M1-specific cells in the memory T cell pool influence the outcome of EBV infection?

I looked for evidence for responses that were preferentially expanded in a primary HIV-specific immune response representing cross-recognition of HIV epitopes by pre-existing memory cells induced to another pathogen. In patient

WEAU, a potential epitope which may have primed the immunodominant HLA-B44-restricted HIV-specific AENLWVTVY(Y) response observed in primary infection in this individual (Borrow *et al.*, 1997) was identified in EBV. This epitope (EENLLDFVRF) was also HLA-B44*03-restricted and shared several amino acids with the HIV epitope, with chemically similar residues at other positions. However when tested, this epitope was found not to be recognised by PBMCs from WEAU. As this approach to studying a potentially cross-reactive response was not optimal (had they been available, cells cryopreserved from the subject prior to HIV infection should have been tested for reactivity to the EBV and HIV epitopes), a HIV seronegative donor known to respond to the EBV epitope was also studied, to see if this response could cross-recognise the HIV epitope. However, no recognition of the HIV epitope was detected in this subject.

As I did not find evidence for cross-reactivity between the EBV EENLLDFVRF response and the HIV AENLWVTVY(Y) response, a search was made for other epitope-specific responses that could have initially primed the response. Viral sequences from viruses which people are commonly infected with, that bore resemblance to the immunodominant HIV epitope AENLWVTVY(Y) were tested for recognition by PBMCs from WEAU in an IFN-γ ELISPOT assay. However, none of the candidate cross-reactive peptides were recognised by patient PBMCs.

A similar approach was adopted to find a viral sequence which might have primed the immunodominant HIV-specific response in patient BORI. Despite the initial finding of two sequences in HSV-2 that were recognised by BORI, follow-up studies revealed that it was likely that the cross-recognition was happening the other way around, with the HIV-specific cells weakly recognising the HSV-2 sequences at high peptide concentrations, suggesting that the HSV-2-specific response did not prime the HIV-specific response.

Another possibility of an example of a cross-recognised peptide came from HIV- and EBV-specific responses studied in patient SC1. This patient was found to have a CD8 response to several known EBV epitopes, including the HLA-B8 EBV epitope RAKFKQLL. It was noticeable that when the relative magnitude of the response to this epitope peptide was measured at two timepoints in acute and then in later infection using IFN-γ ELISPOT assays, the magnitude of the response to the EBV peptide was higher at the later timepoint (data not shown), indicative of expansion of epitope-specific cells as

the HIV-specific CD8 T cell response was induced. There are a number of potential explanations for this finding, but one possibility is that this response was expanded due to cross-recognition of a HIV peptide. Notably, patient SC1 made an immunodominant response to the HLA-B8-restricted HIV epitope FLKEKGGL in primary infection, which shares significant sequence homology with the EBV RAKFKQLL peptide (although when a model was generated to show how they would both look when bound to the HLA-B8 molecule, they did not look very similar). Unfortunately time did not permit cross-reactivity between these epitopes to be explored.

In summary, I was not able to find evidence for $CD8^+$ T cell responses that are expanded rapidly to high levels in acute HIV infection representing expansion of pre-existing memory responses to other pathogens. However, given the vast number of pathogens an individual may encounter during their lifetime, is perhaps not surprising how difficult it is to identify cross-recognised epitopes.

I also tried to look for cross-recognition of HIV epitopes in HIV seronegative individuals. In an attempt to examine this, cells from ten HIV-seronegative subjects were screened for reactivity to overlapping peptides corresponding to the HIV-1 clade B Gag consensus sequence, but no evidence of cross-recognition of HIV Gag peptides was found. This study would need to be extended to a greater number of subjects, looking at a greater proportion of the proteome to give a better idea of the frequency of occurrence of HIV cross-reactivity in HIV seronegative subjects.

There are however, reports in the literature which do support that cross-recognition of HIV in HIV seronegative donors can occur. $CD4^+$ T cell responses to peptides corresponding to the HIV Gag p24 region have been demonstrated in HIV seronegative individuals, suggesting that the responses were due to the priming of such subjects with cross-reactive antigens (Vyakarnam *et al.*, 1991). In addition, the existence of cross-reactivity between HLA-A2 restricted influenza M1:58-66 and HIV-1 p17 Gag:77-85 epitopes was demonstrated following *in vitro* stimulation of PBMC from HIV seropositive or seronegative HLA-A2 donors (Acierno *et al.*, 2003). Together with the finding that *in vitro* stimulation of PBMC from a seronegative donor with the influenza and Gag peptides resulted in expansion of identical T cell clonotypes, this suggested that immunity to the matrix protein of influenza virus may drive a specific immune response to an HLA-A2 restricted HIV Gag epitope in HIV-infected and influenza-immune individuals.

It would be thought that having pre-existing memory cells present would be of value in defence against a pathogen (after all, protection can be conferred by vaccination strategies that elicit virus-specific CD8 T cell responses (Amara *et al.*, 2001; Barouch *et al.*, 2000; Rose *et al.*, 2001; Shiver *et al.*, 2002)). However if, in a situation where APC activation and/or help is deficient, it results in the expansion of a response of extremely limited specificity, it may in fact perhaps be disadvantageous. A highly focused response may be rapidly escaped (Borrow *et al.*, 1997; Jones *et al.*, 2004). It may also be more likely to be exhausted (Pantaleo *et al.*, 1997a). Substantial distortion of the T cell repertoire by monoclonal/oligoclonal T cell expansions during primary HIV infection was observed in patients who went on to establish high but not low persisting viral loads (Pantaleo *et al.*, 1997b). However it is not clear whether these distortions occurred as a consequence of events in acute infection (e.g. high level initial viral replication and associated defects in APC function) and/or played a causative role in determining the persisting viral load established in these individuals.

In summary, in this chapter, mechanisms to account for the rapid initial expansion of small numbers of highly immunodominant responses during primary HIV infection were explored. It was hypothesised that when CD4 help and numbers of fully activated APCs are limiting, there may be an initial expansion of T cell responses that are less dependent on help/co-stimulation, followed by a slower expansion of additional responses. I addressed the ideas that these responses may include high avidity responses and/or responses mediated by HIV-cross-reactive T cells in the memory pool. While further work remains to be done to provide clear-cut answers as to the validity of these two hypotheses, it is possible that both events are occurring.

Expansion of limited numbers of high avidity T cell responses may give rise to the oligoclonal expansions of the T cell Vβ repertoire observed in many HIV-infected patients by Pantaleo *et al* (Pantaleo *et al.*, 1997b). In those patients who have memory T cells that can cross-recognise HIV epitopes, there may be a strong mono-specific response superimposed on this, giving rise to the huge expansion of T cells in a single Vβ family observed by Pantaleo *et al* in ~10% of patients during acute infection.

As emphasised in the introduction to this chapter, the current picture of expansion of the HIV-specific $CD8^+$ T cell responses in primary infection is very incomplete. Studies described in the following chapter aimed to give

more insight into the magnitude/kinetics of expansion of different epitope-specific components of the primary HIV-specific $CD8^+$ T cell response, and the phenotype of the responding cells.

Chapter 5
Characterisation of epitope-specific CD8$^+$ T cell responses in primary HIV-1 infection

5.1 Introduction

During acute viral infections, high levels of viraemia are generally associated with the expansion of virus-specific CD8$^+$ T cells. Precise quantitation of the dynamics of CTL responses in viral infections has been largely restricted to studies in mouse models such as infection with LCMV. During LCMV infection, the virus-specific CD8$^+$ T cell response peaks just after the peak of viral replication, at around day 8 post-infection, with virus-specific CD8$^+$ T cells comprising >50% of all splenic CD8$^+$ T cells at this time (Murali-Krishna *et al.*, 1998), then declines as the viral load falls. Studies of the dynamics of the virus-specific CD8$^+$ T cell response during primary HIV infection have been much more limited. These have indicated that HIV-specific CD8$^+$ T cell responses are expanded as the acute burst of viral replication occurs (Borrow *et al.*, 1994; Koup *et al.*, 1994), and can potentially reach very high magnitudes (Borrow *et al.*, 1997; Pantaleo *et al.*, 1994); however the dynamics of expansion of epitope-specific components of the response have not been well characterised. Limited analysis of the dynamics of responses to just one or two viral epitopes in one-three patients has been reported (Appay, 2002; Wilson *et al.*, 2000), but in some cases, the T cell response was quantified at timepoints when the subject was receiving antiretroviral therapy, which likely influenced the picture obtained. Furthermore, multiple epitope-specific responses were not studied, and it was not known whether the responses which were tracked were the initial and/or most dominant responses within the patient's entire primary HIV-specific response. Therefore many questions remain unanswered about the primary HIV-specific CD8$^+$ T cell response. For example, it is unclear how large the total HIV-specific CD8$^+$ T cell response expanded is; and what the kinetics are with which initial epitope-specific response(s) expand and decline, or how they relate to the kinetics of the acute burst of viral replication.

In chapter 3, I used IFN-γ ELISPOT assays to assess the 'entire' HIV-specific CD8$^+$ T cell response during early infection in a number of individuals. Although ELISPOT assays allow for easy and rapid identification of responses, they have limitations, for example they are not very quantitative. However the identification of optimal HIV CD8$^+$ epitopes and their restricting

HLA alleles in chapter 3 allowed for the use of peptide-MHC tetramers for more in-depth analysis of individual epitope-specific responses. Tetramers have the advantage of being able to detect all T cells specific for an epitope, regardless of function, and therefore magnitudes of responses can be determined more accurately using this technique. In this chapter, I used tetramers specific for different epitopes within a patient's response to address the kinetics of expansion of T cells of different specificities, by staining cells cryopreserved over a series of timepoints during acute and early infection and determining the size of the tetramer-positive population at each timepoint.

Another aspect of the HIV-specific $CD8^+$ T cell response which was explored in chapter 3 was the repertoire of T cells used within an individual response. In chapter 3, this was determined using cells from a single timepoint. How the repertoire of T cells used in an epitope-specific response might alter throughout the course of infection (for example, do changes in the T cell repertoire reflect affinity maturation of the epitope-specific T cell response?) is another question of interest. Therefore, in this chapter, as an extension to the kinetic analysis of individual epitope-specific responses, the Vβ family usage by T cells responding to an individual epitope at different timepoints was also determined.

By co-staining tetramer-positive cells with antibodies to other surface and intracellular molecules, phenotypic characterisation of HIV-specific cells can be performed. There have been many phenotypic studies of HIV-specific $CD8^+$ T cells, which have been carried out to address qualitative aspects of the response and whether these may be related to its ability to control viral replication. It has been suggested that a high proportion of the HIV-specific $CD8^+$ T cells present in chronically-infected patients may not be fully functional. Many of these cells are reported to have an 'immature' phenotype ($CCR7^-$ $CD27^+$ $CD45RA^-$) suggestive of incomplete maturation (Appay *et al.*, 2000; Champagne *et al.*, 2001), to contain low levels of perforin, and to exhibit low *ex vivo* IL-2 production and proliferative capacity (Appay *et al.*, 2000; Appay *et al.*, 2002; Champagne *et al.*, 2001; Migueles *et al.*, 2002; van Baarle *et al.*, 2002a). Furthermore, some (Goepfert *et al.*, 2000; Kostense *et al.*, 2001; Shankar *et al.*, 2000) but not other studies (Goulder *et al.*, 2000) have found deficits in the ability of HIV-specific $CD8^+$ T cells to produce antiviral cytokines. These studies have been largely limited to chronically-infected patients, so it is not clear whether dysfunctional HIV-specific cells are also present in acute infection (although the expression of CD27 on a large

proportion of HIV-specific $CD8^+$ T cells has also been found to be a feature of primary infection (Appay, 2002; Papagno *et al.*, 2004)).

In this chapter the maturation state of the T cells responding to different viral epitopes during acute and early HIV infection was examined, by analysing the phenotype (expression of CCR7, CD27, CD28 and CD45RA) of tetramer-positive cells, to reveal whether defects in the maturation state of HIV-specific cells are present from primary infection, and/or whether these defects are acquired over time. Other markers of interest were also examined to obtain information about the replicative capacity of the cells and their ability to respond to stimulatory cytokines. T cells responding to different (immunodominant and subdominant) viral epitopes were studied, to determine whether they had similar phenotypic characteristics; further, this type of analysis was carried out on cells from patients who established different persisting viral loads to determine whether there were any qualitative differences in the $CD8^+$ T cell response in patients who control viral replication with differing efficiency.

The overall aim of the work in this chapter thus was to further characterise the HIV-specific $CD8^+$ T cell response during primary infection with the use of tetramers, to build upon the initial observations about the response made in the previous chapters.

5.2 Analysis of the magnitude and kinetics of expansion of $CD8^+$ T cells responding to different HIV epitopes during primary infection.

Early reports revealed that HIV-specific $CD8^+$ T cell responses can be induced very early on in infection (Borrow *et al.*, 1994; Koup *et al.*, 1994; Pantaleo *et al.*, 1994). However the techniques used in these studies did not allow for accurate quantitation of the expansion and contraction of epitope-specific responses. The later availability of tetramers has allowed for more accurate assessment of the kinetics of HIV epitope-specific responses, but only limited studies have been carried out using this technology (Appay, 2002; Wilson *et al.*, 2000), and thus many questions remain unanswered. For example, it has yet to be established whether the T cells responding to different epitopes expand with similar kinetics, or whether some responses evolve more slowly than others. To address this question, PBMCs from different HIV-infected individuals cryopreserved at sequential timepoints during acute and early infection were stained using MHC-peptide tetrameric (or pentameric) complexes to determine the magnitude of epitope-specific $CD8^+$ T cell

responses at each timepoint and reveal the dynamics of expansion and decline of responses to different viral epitopes.

The epitope-specific responses to be studied in this way were chosen on the basis of results obtained in chapter 3. Patients were selected in whom two or three dominant and subdominant responses had been identified to epitopes that it was possible to obtain tetramers/pentamers for. I also tried to include patients who established different persisting viral loads, so that it would be possible to determine whether there were any differences in the rate of expansion of responses in patients who controlled early viral replication with differing efficiency.

Altogether, responses were studied in one patient who established a high persisting viral load (MM25), three who established intermediate-high persisting viral loads (MM12, MM27 and MM14) and two patients who established low-intermediate persisting viral loads (MM13 and MM28). It should be noted that the epitope-specific responses studied in each patient did not represent the total HIV-specific response of that patient, only selected epitope-specific responses for which tetramers could be obtained.

PBMC cryopreserved at sequential timepoints during acute and early infection were co-stained with the appropriate tetramer or pentamer, an anti-CD3 antibody and an anti-CD8 antibody, and the frequency of $CD8^+$ T cells specific for the epitope determined by flow cytometry by calculating the percentage of $CD8^+$ $tetramer^+$ cells when acquired events were gated on the $CD3^+$ $CD8^+$ lymphocyte population. Representative dot plots from one such timecourse of staining are shown in Figure 5.1, where the frequency of $CD8^+$ T cells specific for the A3 RLRPGGKKK epitope was determined in samples cryopreserved from patient MM25 at sequential timepoints from acute/subacute infection until the time when the viral setpoint was established. The proportion of $CD8^+$ cells that were $tetramer^+$ was high (3.3%) at the first timepoint investigated (17 DFOSx) and increased to a peak of 6.2% at 31 DFOSx. Thereafter, the response declined in magnitude, but there was still a sizeable proportion of cells stained by this tetramer at the latest timepoint studied (2.1% at 185 DFOSx). This example illustrates that the magnitude of an individual HIV-specific response can be quite large at its peak – over 6% of $CD8^+$ cells in the case of this response. Given that this is not the only epitope that was recognised in patient MM25's early HIV-specific response (it can be seen in Figure 5.2a that during early infection there were strong responses to three additional epitopes, plus a number of more minor responses), the total

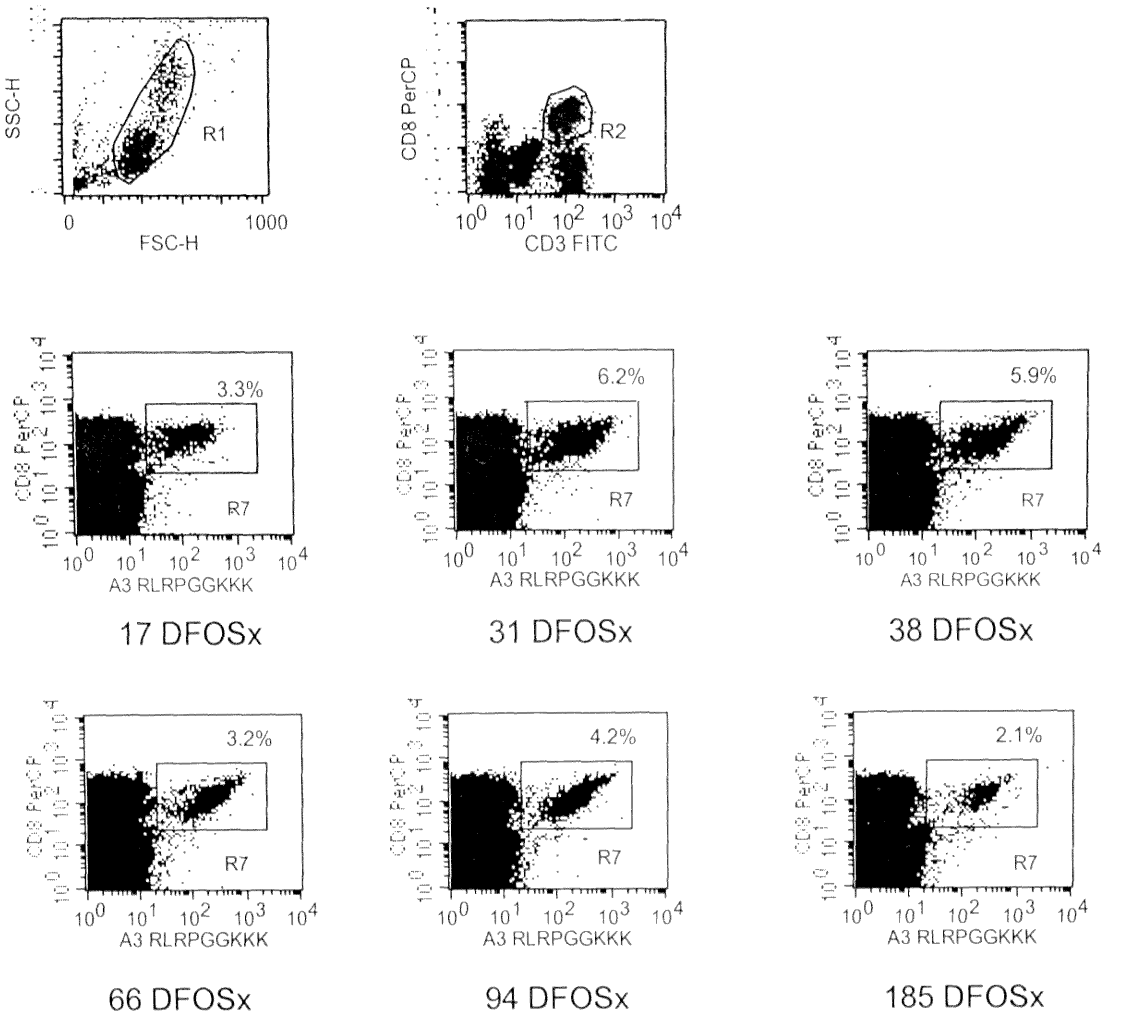


Figure 5.1. Analysis of the magnitude of the HIV A3 RLRPGGKKK-specific CD8$^+$ T cell response over the course of infection in patient MM25. PBMC cryopreserved from patient MM25 at sequential timepoints during the course of infection were co-stained with PE-conjugated A3 RLRPGGKKK tetramer, PerCP-conjugated anti-CD8 and FITC-conjugated anti-CD3 antibodies. The frequency of CD8$^+$ cells specific for the epitope at each timepoint was determined by flow cytometry. Dot plots of the staining obtained using cells from each timepoint are shown. Each plot is gated on the CD3$^+$ CD8$^+$ lymphocyte population shown in R2. The percentage of CD8$^+$ tetramer$^+$ cells at each timepoint is given in the relevant dot plot.

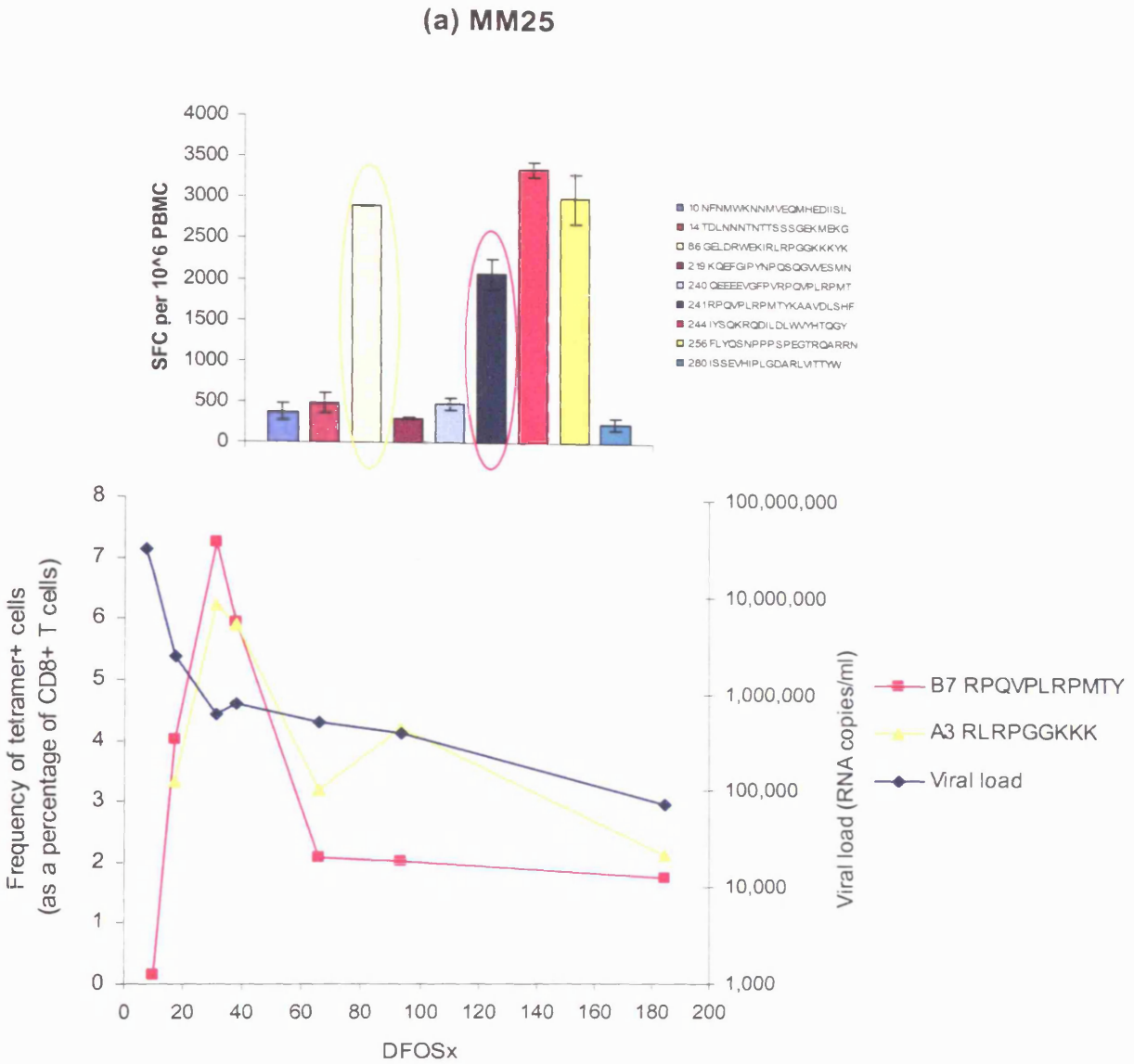
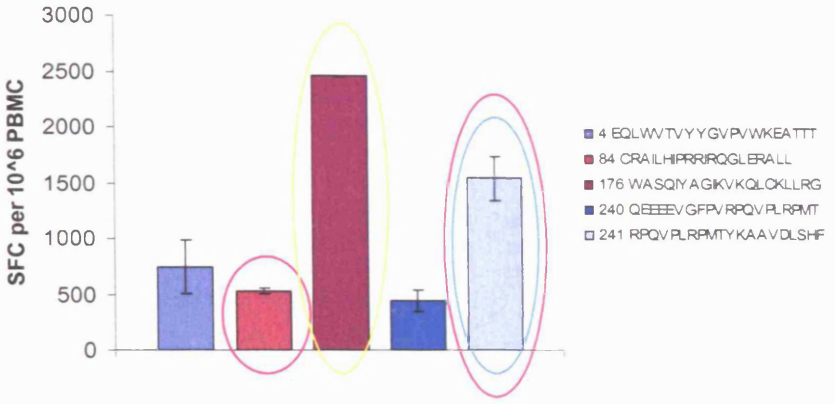
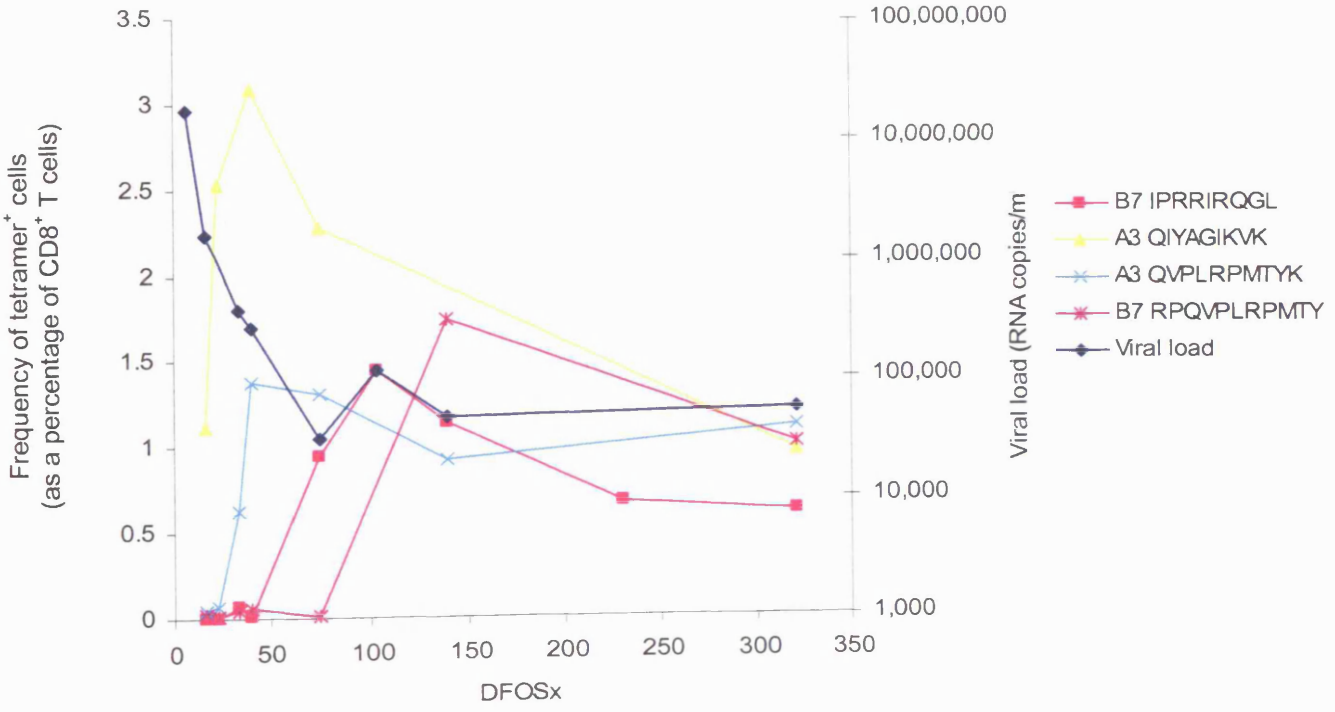


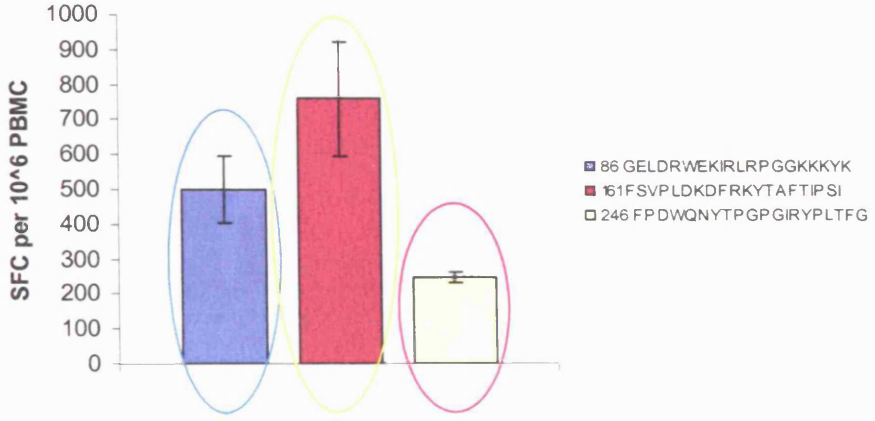
Figure 5.2. Analysis of the magnitude and kinetics of expansion of HIV-specific CD8$^+$ T cell responses within HIV-infected individuals who established different persisting viral loads. Long peptides corresponding to HIV epitopic regions shown to be recognised by the early HIV-specific CD8$^+$ T cell response of selected patients, and the magnitude of the response they elicited in an IFN-γ ELISPOT assay (determined as described in Figures 3.11-3.13) are shown in the upper half of part of the figure. HIV epitopes were predicted within the selected long peptides circled in the bar chart and CD8$^+$ T cell responses targeting predicted epitopes were further characterised. PBMCs cryopreserved from seven HIV seroconvertors at sequential timepoints over the course of primary HIV infection were co-stained with antibodies to CD3, CD8 and MHC class I tetramers or pentamers corresponding to HIV epitopes. The percentage of CD8 cells stained with each tetramer/pentamer at each timepoint is plotted alongside viral load data to illustrate the expansion of each response relative to the viral load for (a) MM25, a patient who established a high persisting viral load, (b) MM12, MM27 and MM14, patients who established intermediate-high persisting viral loads, (c) MM13 and MM28, patients who established low-intermediate persisting viral loads, and (d) SUMA, a patient who established a low persisting viral load. N.B. HIV epitopes were identified using a different approach for patient SUMA compared to that used for other patients, and so comparable data about the entire early response is not available. The data in part (d) of this figure were generated by Dr N. A. Jones.

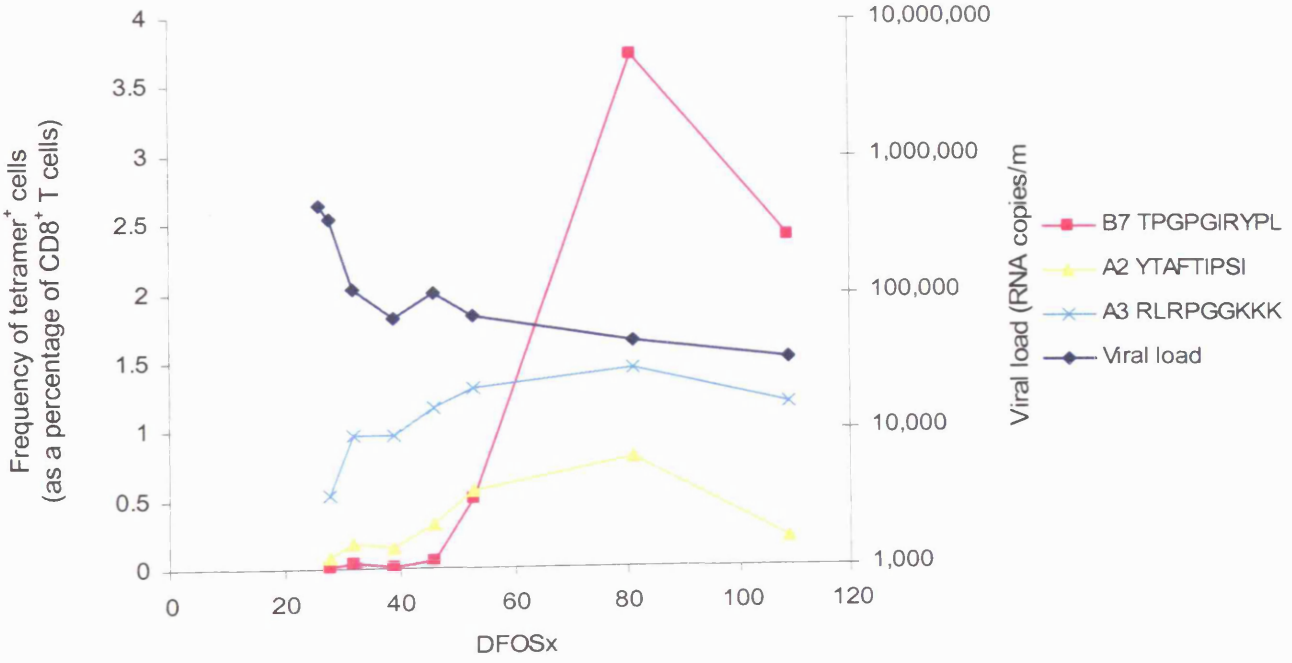
(b) (*i*) MM12


SFC per 10^6 PBMC
3000
2500
2000
1500
1000
500
0
4 EQLWVTVYYGVPVWKEATTT
84 CRAILHIPRRIRQGLERALL
176 WASQIYAGIKVKQLCKLLRG
240 QEEEEVGFPVRPQVPLRPMT
241 RPQVPLRPMTYKAAVDLSHF

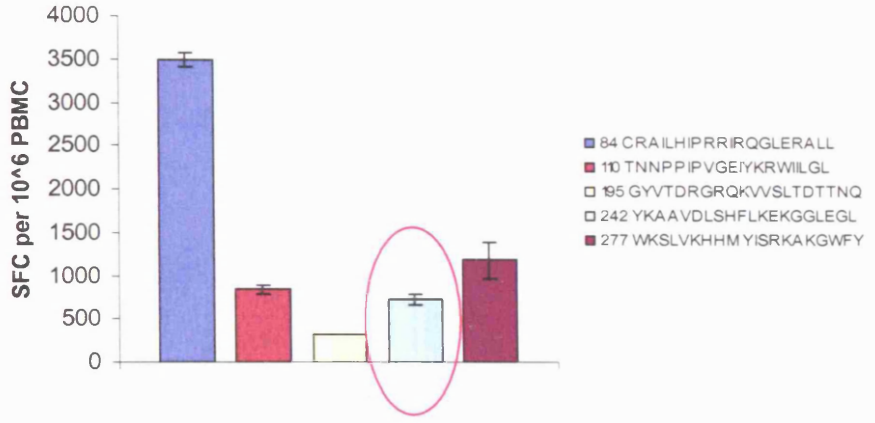

Frequency of tetramer+ cells
(as a percentage of CD8+ T cells)
3.5
3
2.5
2
1.5
1
0.5
0
0
50
100
150
200
250
300
350
DFOSx
100,000,000
10,000,000
1,000,000
100,000
10,000
1,000
Viral load (RNA copies/m
B7 IPRRIRQGL
A3 QIYAGIKVK
A3 QVPLRPMTYK
B7 RPQVPLRPMTY
Viral load

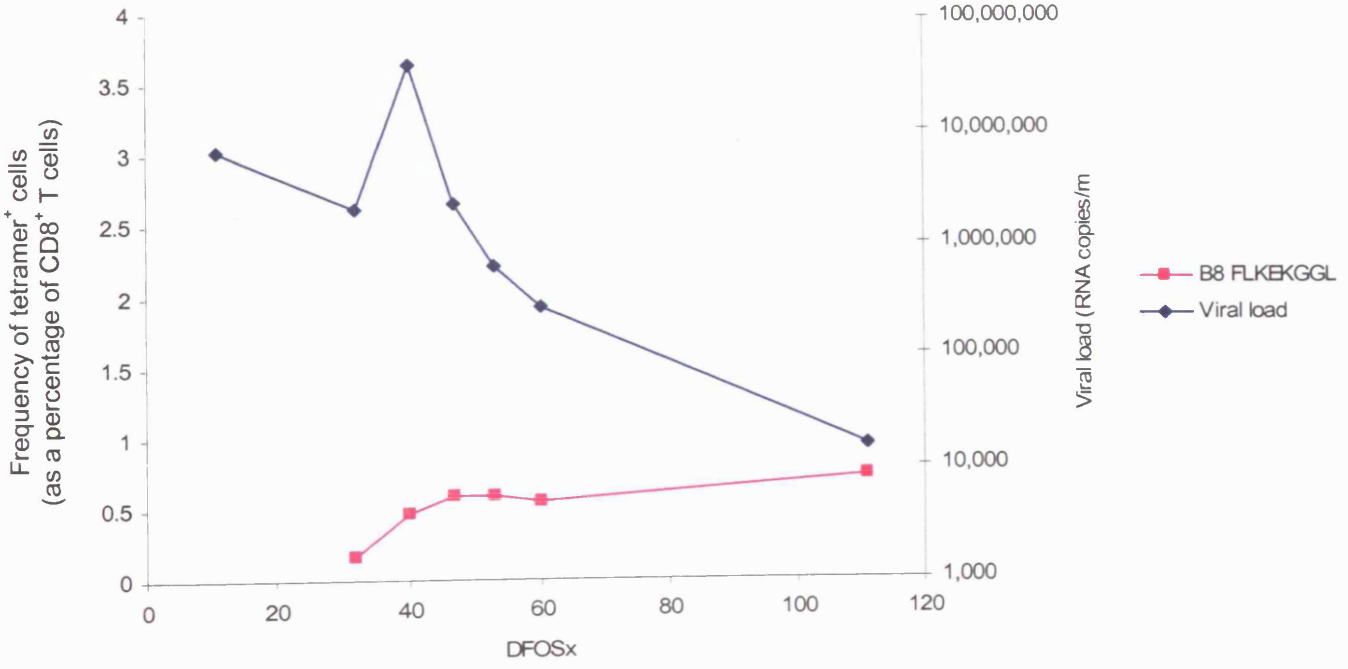
(b) (*ii*) MM27


SFC per 10^6 PBMC
1000
900
800
700
600
500
400
300
200
100
0
86 GELDRWEKIRLRPGGKKKYK
161FSVPLDKDFRKYTAFTIPSI
246 FPDWQNYTPGPGIRYPLTFG

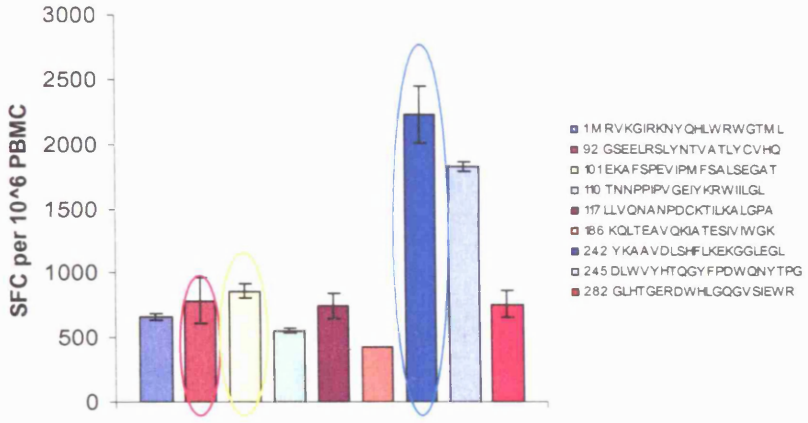

Frequency of tetramer+ cells
(as a percentage of CD8+ T cells)
4
3.5
3
2.5
2
1.5
1
0.5
0
0
20
40
60
80
100
120
DFOSx
10,000,000
1,000,000
100,000
10,000
1,000
Viral load (RNA copies/m
B7 TPGPGIRYPL
A2 YTAFTIPSI
A3 RLRPGGKKK
Viral load

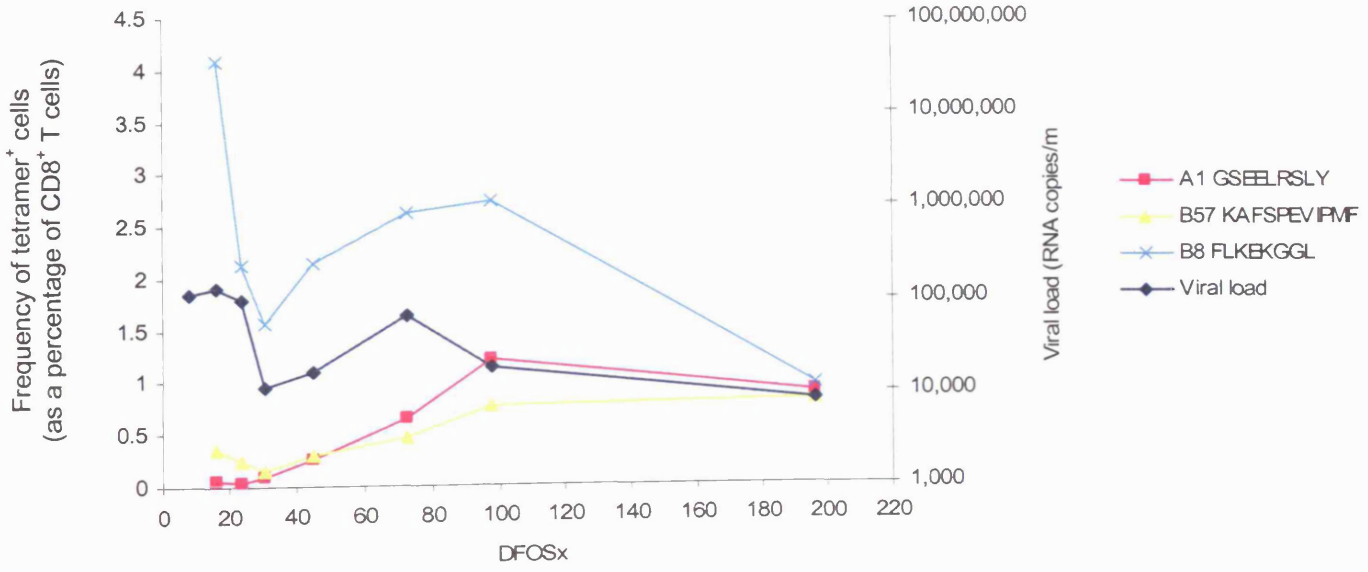
(b) (*iii*) MM14


SFC per 10^6 PBMC
4000
3500
3000
2500
2000
1500
1000
500
0
84 CRAILHIPRRIRQGLERALL
110 TNNPPIPVGEIYKRWIILGL
195 GYVTDRGRQKVVSLTDTTNQ
242 YKAAVDLSHFLKEKGGLEGL
277 WKSLVKHHMYISRKAKGWFY

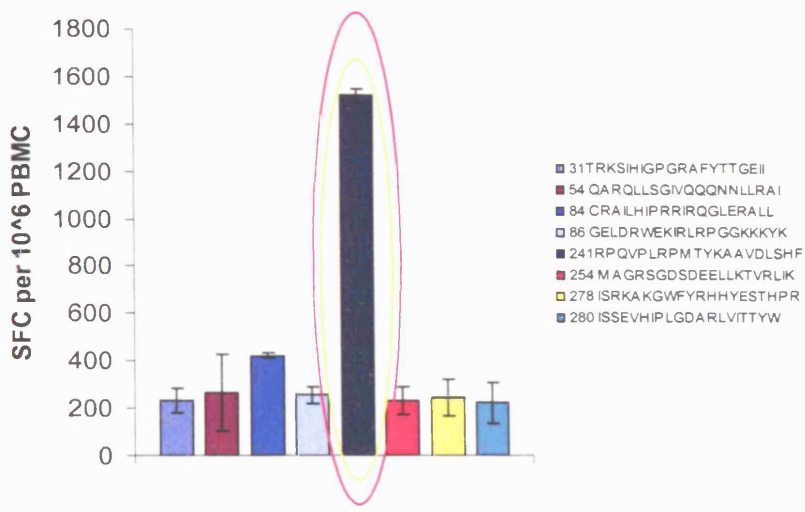

Frequency of tetramer+ cells
(as a percentage of CD8+ T cells)
4
3.5
3
2.5
2
1.5
1
0.5
0
0
20
40
60
80
100
120
DFOSx
100,000,000
10,000,000
1,000,000
100,000
10,000
1,000
Viral load (RNA copies/m
B8 FLKEKGGL
Viral load

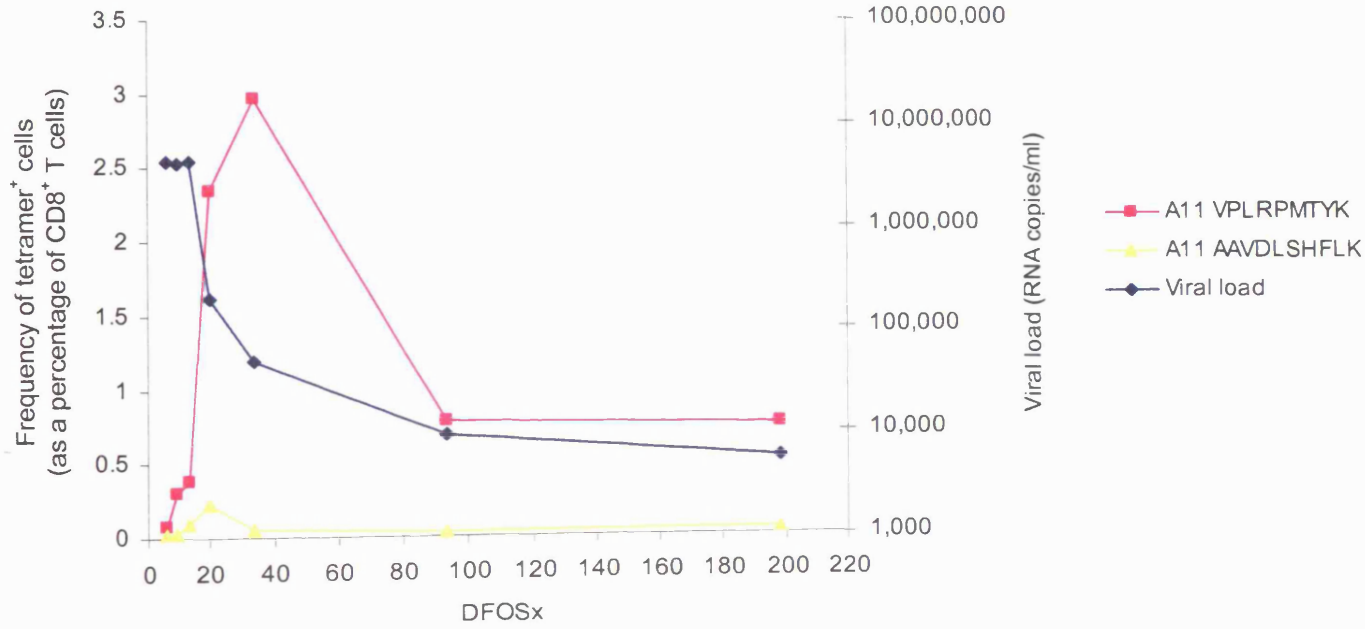
(c) (*i*) MM13


SFC per 10^6 PBMC
3000
2500
2000
1500
1000
500
0
1 M R V K G I R K N Y Q H L W R W G T M L
92 G S E E L R S L Y N T V A T L Y C V H Q
101 E K A F S P E V I P M F S A L S E G A T
110 T N N P P I P V G E I Y K R W I I L G L
117 L L V Q N A N P D C K T I L K A L G P A
186 K Q L T E A V Q K I A T E S I V I W G K
242 Y K A A V D L S H F L K E K G G L E G L
245 D L W V Y H T Q G Y F P D W Q N Y T P G
282 G L H T G E R D W H L G Q G V S I E W R

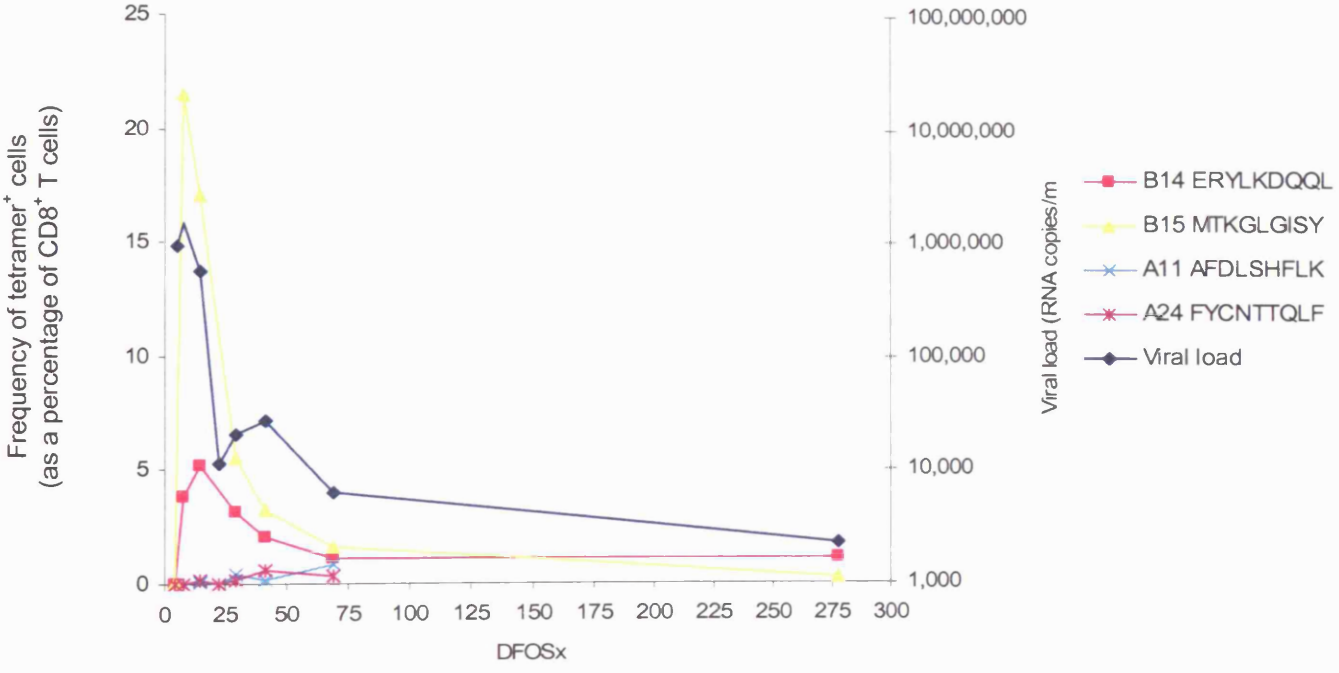

Frequency of tetramer+ cells
(as a percentage of CD8+ T cells)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
20
40
60
80
100
120
140
160
180
200
220
DFOSx
100,000,000
10,000,000
1,000,000
100,000
10,000
1,000
Viral load (RNA copies/m
A1 GSEELRSLY
B57 KAFSPEVIPMF
B8 FLKEKGGL
Viral load

(c) (*ii*) MM28


SFC per 10^6 PBMC
1800
1600
1400
1200
1000
800
600
400
200
0
31TRKSIHIGPGRAFYTTGEII
54 QARQLLSGIVQQQNNLLRAI
84 CRAILHIPRRIRQGLERALL
86 GELDRWEKIRLRPGGKKKYK
241RPQVPLRPMTYKAAVDLSHF
254 MAGRSGDSDEELLKTVRLIK
278 ISRKAKGWFYRHHYESTHPR
280 ISSEVHIPLGDARLVITTYW


Frequency of tetramer+ cells
(as a percentage of CD8+ T cells)
3.5
3
2.5
2
1.5
1
0.5
0
0
20
40
60
80
100
120
140
160
180
200
220
DFOSx
100,000,000
10,000,000
1,000,000
100,000
10,000
1,000
Viral load (RNA copies/ml)
A11 VPLRPMTYK
A11 AAVDLSHFLK
Viral load

(d) SUMA


Frequency of tetramer+ cells
(as a percentage of CD8+ T cells)
25
20
15
10
5
0
0
25
50
75
100
125
150
175
200
225
250
275
300
DFOSx
100,000,000
10,000,000
1,000,000
100,000
10,000
1,000
Viral load (RNA copies/m
B14 ERYLKDQQL
B15 MTKGLGISY
A11 AFDLSHFLK
A24 FYCNTTQLF
Viral load

magnitude of the primary HIV-specific $CD8^+$ T cell response is potentially very high.

Other tetramers were used in a similar manner to analyse the magnitude of epitope-specific responses over time in this and other patients. The results obtained are shown in Figure 5.2.

In addition to the RLRPGGKKK-specific response mounted by MM25 in early infection, the B7-restricted epitope RPQVPLRPMTY was also targeted. The responses to the long peptides containing these epitopes as measured by IFN-γ ELISPOT assay were both relatively strong within this patient's early response. In Figure 5.2a, the magnitude of the response made to these two epitopes over a series of timepoints, as revealed by tetramer staining, has been plotted. It can be seen that the T cells specific for the two epitopes expanded simultaneously, rising up to a peak of around 6-8% of $CD8^+$ cells at 31 DFOSx and then declining to an almost steady level by 66 DFOSx. As the viral load was $>10^7$ RNA copies/ml at 7 DFOSx, it is likely that this was (or was close to) the timepoint at which the acute burst of viral replication reached its peak. If the peak of viraemia is taken as being at 7 DFOSx (the timepoint at which the highest viral load was recorded) the two responses studied reached their peak magnitude between one and three weeks following the peak in viral load (the possibility that the responses peaked between 17 and 31 DFOSx cannot be excluded as there were no samples available for testing between these two timepoints).

The evolution of four responses was followed in patient MM12 (Figure 5.2bi); these exhibited a somewhat different pattern of expansion than that of the responses studied in patient MM25 (although this may be simply be due to a more limited number of responses being studied in patient MM25). The epitopes to which responses were studied were located within three long peptides used for mapping the early response, as one of the long peptides was found to contain two epitopes restricted by different HLA alleles. It can be seen that the most dominant response in early infection was made to the A3-restricted epitope QIYAGIKVK, with a relatively strong response also being made to the long peptide containing the A3 QVPLRPMTYK and B7 RPQVPLRLMTY epitopes (although the relative dominance of responses made to the two individual epitopes is unknown). B7 IPRRIRQGL represents a subdominant epitope within this patient's early HIV-specific response. Unlike the two responses studied in patient MM25, the four responses studied in MM12 did not expand in parallel, but rather at different rates, with two faster

and two slower evolving responses. This patient's peak viral load probably occurred at around 7 DFOSx. The first of the four responses studied to expand was the A3 QIYAGIKVK-specific response, which was in fact the most dominant of the responses detected in this patient in early infection. T cells targeting this epitope were detected at 16 DFOSx, just over a week after the highest recorded viral load, and the response peaked at around 40 DFOSx, at around 3% of $CD8^+$ cells. A lower magnitude response was raised against the A3 QVPLRPMTYK epitope. This response expanded with similar kinetics to the A3 QIYAGIKVK epitope. The two other responses studied were slower to emerge, and were only detected as the other two faster evolving responses were declining, after 75 DFOSx. The B7 IPRRIRQGL-specific response peaked at 104 DFOSx, while at 139 DFOSx, the B7 RPQVPLRLMTY-specific response was the slowest of the four responses studied to peak.

Responses were detected to only three long peptides in patient MM27 in early infection (Figure 5.2bii), and tetramers corresponding to epitopes in all three long peptides were obtained. It was not possible to precisely relate the kinetics of T cell expansion in this patient to the kinetics of acute viral replication, as the first timepoint at which the viral load was measured in this patient was likely well after the peak in viraemia. Nonetheless, the three responses all expanded with delayed kinetics, not reaching their peak levels until 81 DFOSx. However, it was feasible that there could have been response(s) that emerged earlier that were not identified in the mapping of this patient's early response. It is interesting that the relative magnitude of the responses to the three epitopes as measured by tetramer staining does not reflect the relative magnitude of the responses to the long epitope-containing peptides measured by IFN-γ ELISPOT assay at 53 DFOSx. Notably, the response to the long peptide containing the A2 YTAFTIPSI epitope appeared most dominant in the ELISPOT assay, whereas the tetramer recognising T cells responsive to this epitope bound a lower number of $CD8^+$ T cells than the A3 RLRPGGKKK tetramer. This might be explained by the tetramer not corresponding to the optimal epitope within the most dominant long peptide. Two variants of the same epitope were possible, one a 10 amino acid sequence, and the other a 9 amino acid sequence. When peptides corresponding to the two possible epitopes were tested by IFN-γ ELISPOT, the response detected to the 10mer was of slightly higher magnitude than that to the 9mer. However, due to technical reasons, it was only possible to have the tetramer synthesised using the 9mer sequence, which might have influenced the relative magnitude of

response detected using this tetramer. Another possible explanation for why the dominance of the long peptide containing the A2 YTAFTIPSI epitope in the ELISPOT assay was not reflected by observations made by use of an A2 YTAFTIPSI tetramer, was that the dominant long peptide may have contained an additional epitope that was not identified.

The B8 FLKEKGGL-specific response represented one subdominant component within patient MM14's early HIV-specific $CD8^+$ response (Figure 5.2biii). It was not possible to obtain tetramers suitable for following the other epitope-specific responses mapped in this patient. It was not quite so clear in this case as to when the epitope-specific T cell response peaked relative to the peak in viral load as it was hard to establish when the peak in viral replication occurred. The highest recorded viral load was at 40 DFOSx; but it seems likely (based on observations made in other patients) that the peak in viral load was reached considerably before this, with the fall and then apparent rise in viral load between 11 and 40 DFOSx representing fluctuations in viraemia as viral replication was being contained. Figure 5.2biii shows that T cells specific for FLKEKGGL were detected from the earliest timepoint tested (32 DFOSx), and reached an initial maximum frequency by 47 DFOSx. This frequency was maintained (or slightly increased) at the last timepoint tested (111 DFOSx).

Tetramers were obtained to enable one dominant (B8 FLKEKGGL) and two subdominant (A1 GSEELRSLY and B57 KAFSPEVIPMF) epitope-specific responses to be tracked in patient MM13. It can be seen from the viral load data plotted in Figure 5.2ci that this patient was likely enrolled onto the study after the peak in primary viraemia had already occurred, as the highest recorded viral load was only 131 800 RNA copies/ml, whereas peak viraemia is typically 10^6-10^8 RNA copies/ml. T cells specific for all four epitopes were detected from the first timepoint where cells were available for study (16 DFOSx), but the responses subsequently evolved in different ways. The dominant FLKEKGGL-specific response was already at its highest recorded magnitude at the first timepoint tested, suggesting that this response emerged very rapidly. The response quickly declined, and the magnitude of the response at the next timepoint (24 DFOSx) was only half of what it was at 16 DFOSx. Although the general trend was that of a decline from 16 DFOSx onwards, there was an increase in the magnitude of the response between 31 and 98 DFOSx, which interestingly paralleled the fluctuation of the viral load at this time, suggesting that viral rebound might have led to the expansion of this

immunodominant response once more. By contrast to the dominant response, responses to the subdominant epitopes were much slower to reach their peak. The response to the GSEELRSLY epitope peaked at 98 DFOSx, whilst that to KAFSPEVIPMF was still rising at the last timepoint studied (unrelated studies done using cells from later timepoints revealed that the response may have peaked as late as 1125 DFOSx).

A11 tetramers were obtained containing two different sequences (VPLRPMTYK and AAVDLSHFLK) which could both represent epitopes within the same long peptide (RPQVPLPMTYKAAVDLSHF), to which a dominant response was observed in patient MM28 in early infection (Figure 5.2cii). This patient's peak viral load probably occurred at (or prior to) 6 DFOSx; responses to the two tetramers emerged soon after this, although they expanded at slightly different rates. The smaller of the two responses (AAVDLSHFLK) peaked at 20 DFOSx, approximately two weeks following the peak in viral load (although the low magnitude of the response to this epitope at all timepoints perhaps makes it difficult to be certain about this). The peak of the larger of the two responses (VPLRPMTYK) (around 3% of $CD8^+$ cells) was recorded at 34 DFOSx, approximately four weeks after the viral load likely peaked.

One limitation of the London cohort was that there were no patients available who established low persisting viral loads. However there was one such patient (patient SUMA) from another patient cohort. Epitope-specific responses in patient SUMA had been followed by tetramer staining throughout the course of acute and early infection by Dr N. A. Jones (unpublished data) – the results of this analysis are shown in Figure 5.2d, and are included in all subsequent analyses (where data from multiple patients are combined) so that the analysis includes patients who established persisting viral loads falling into all four viral load quartiles.

The primary HIV-specific $CD8^+$ T cell response mounted by patient SUMA was directed against >40 epitopes, with the most dominant response being that to the B15–restricted MTYKGLGISY epitope, and other responses bring of lower magnitude and more co-dominant. Four epitope-specific responses were followed in patient SUMA: the dominant B15 MTYKGLGISY-specific response, and three less dominant responses, B14 ERYLKDQQL, A11 AFDLSHFLK and A24 FYCNTTQLF. In Figure 5.2d it can be seen that the response detected to B15 MTYKGLGISY by tetramer staining was of considerable magnitude, with >20% of CD8 cells being specific for this epitope at the peak of the response. Furthermore, the MTYKGLGISY-specific response expanded with extremely

rapid kinetics: the highest recorded magnitude response occurred very early (between 0 and 7 days) after the highest recorded viral load (recorded at 8 DFSOx). A sizeable response was also detected towards B14 ERYLKDQQL, and this response was also one which was quick to expand, reaching > 5% of $CD8^+$ T cells at 15 DFOSx. The two other epitope-specific responses followed (A11 AFDLSHFLK and A24 FYCNTTQLF) were of lower magnitude (<1 % of the $CD8^+$ T cells at all timepoints studied) and expanded with slower kinetics relative to the two more dominant epitope-specific responses. The highest magnitude responses to these epitopes were recorded at 69 and 41 DFOSx respectively, but it is possible that these responses may have continued to rise, as timepoints beyond 69 DFOSx were not tested using these particular tetramers.

Considering all epitope-specific responses studied in all patients, the highest peak response observed to a single viral epitope was almost 22% of $CD8^+$ cells (the MTYKGLGISY-specific response in patient SUMA); other responses generally peaked at 7% or less of $CD8^+$ T cells. The minimal size of individual responses was around the level of detection of this technique, at 0.01% of $CD8^+$ cells. When the magnitude of the individual responses studied within each patient were combined, the highest total HIV-specific CD8 response was that seen in patient SUMA, with 26.7% of CD8 cells being directed towards just two epitopes. The next largest combined response was that observed in patient MM25 in whom 13.5% of CD8 cells were directed towards two epitopes. These values provide an underestimate of the magnitude of the entire HIV-specific $CD8^+$ T cell response in the individuals concerned, as each also mounted responses to additional epitopes that were not included in the analysis. It would be unfair to comment on minimal size total responses as in some patients, responses to only a limited number of epitopes were studied, and these did not constitute the most dominant components of the patient's primary HIV-specific response.

The kinetics of expansion of different epitope-specific responses was quite variable, as can be seen when all responses are plotted together on a graph of the magnitude of the response against the time from the estimated peak viral load (defined as the day at which the highest viral load was recorded, or was estimated to have occurred in those patients where it was likely that the peak in viraemia had been missed) (Figure 5.3). The time at which the peak in the epitope-specific response occurred relative to the estimated peak in viral load varied between 0 and 189 days. However, of the 19 responses studied,

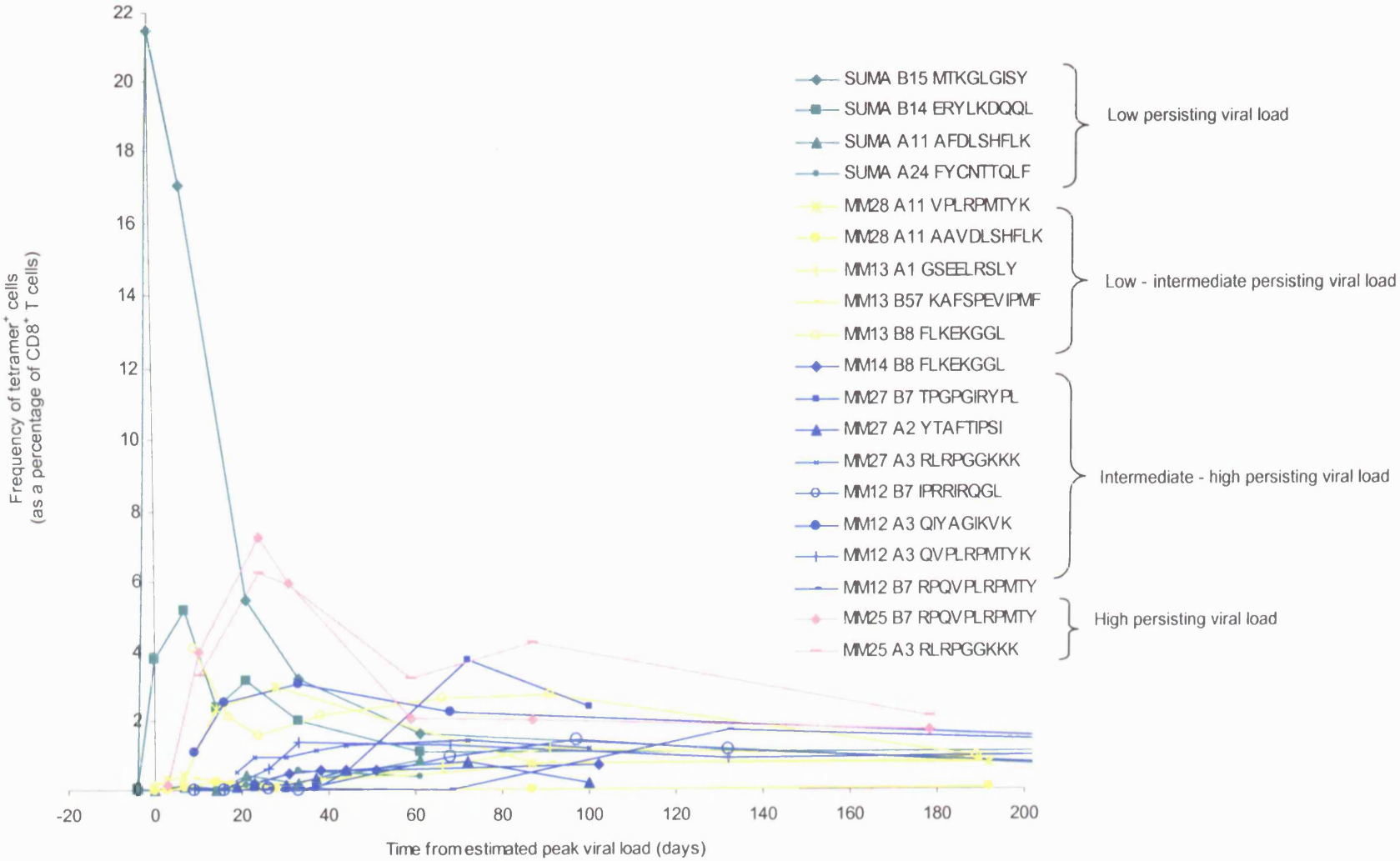


Figure 5.3. Variation in the kinetics of expansion of epitope-specific CD8$^+$ T cell responses in HIV-infected patients. PBMC cryopreserved over the course of acute, early (and later) infection from patients SUMA, MM28, MM13, MM14, MM27, MM12 and MM25 were stained with tetramers/pentamers corresponding to HIV epitopes identified to be recognised by the early HIV-specific CD8$^+$ T cell response of the patient (as indicated in the key), and antibodies specific for CD3 and CD8. The percentage of CD8$^+$ tetramer$^+$ cells at each timepoint was determined by flow cytometry and is plotted above against the time from the estimated peak viral load that the cells were cryopreserved. Different responses are color-coded in the graph to indicate the persisting viral load established by the patient in which the response was followed (as indicated in the key).

only 2 (both in patient SUMA) peaked within a week of the estimated peak in viral load; the remainder took a minimum of 14 days to reach their peak. Almost half of the responses studied reached their maximum recorded level within 33 days after the estimated peak in viral load.

Considering the expansion of responses to different epitopes within individual patients, responses generally expanded asynchronously, with some faster and some slower evolving responses within each patient. This was the case for 4 of the 6 patients in which more than one epitope-specific response was studied. However in two cases, responses expanded in parallel (in patient MM25 the maximum recorded magnitude of the two epitope-specific responses studied occurred 24 days after the estimated peak in viral load; in patient MM27, three responses peaked after 72 days). However, this analysis is complicated by the fact that it is not certain that all responses in early infection were followed for all patients, so there may have been other responses not studied in these two patients that expanded asynchronously relative to the others.

One question of interest is whether a relationship exists between the kinetics of expansion of a response and its magnitude, for example, do larger responses peak earlier? To address this question, for all epitope-specific responses followed in all patients studied, the DFOSx at which the highest magnitude response was recorded was plotted against the highest recorded magnitude of the response (Figure 5.4). Combining the data from all epitopic responses revealed that there was an inverse correlation between the magnitude and kinetics of responses; this relationship was found to be statistically significant using Pearson's correlation test ($p=0.04$).

A further question of interest is whether responses in patients who control viral replication with differing efficiency differ in terms of magnitude and kinetics of expansion. With respect to the magnitude of responses, similar to results obtained in chapter 3 using IFN-γ ELISPOT assays to assess responses, it was found that the magnitude of HIV-specific CD8 responses can be large in patients who control early viral replication with efficiencies at both ends of the spectrum; high magnitude responses were observed in both patient SUMA and patient MM25, who controlled early viral replication well and poorly respectively. What is interesting is the kinetics with which responses were expanded in patient SUMA compared to the other patients studied: the most dominant response in patient SUMA reached its highest recorded value rapidly relative to the estimated peak in viral load (between 0 and 7 days); and

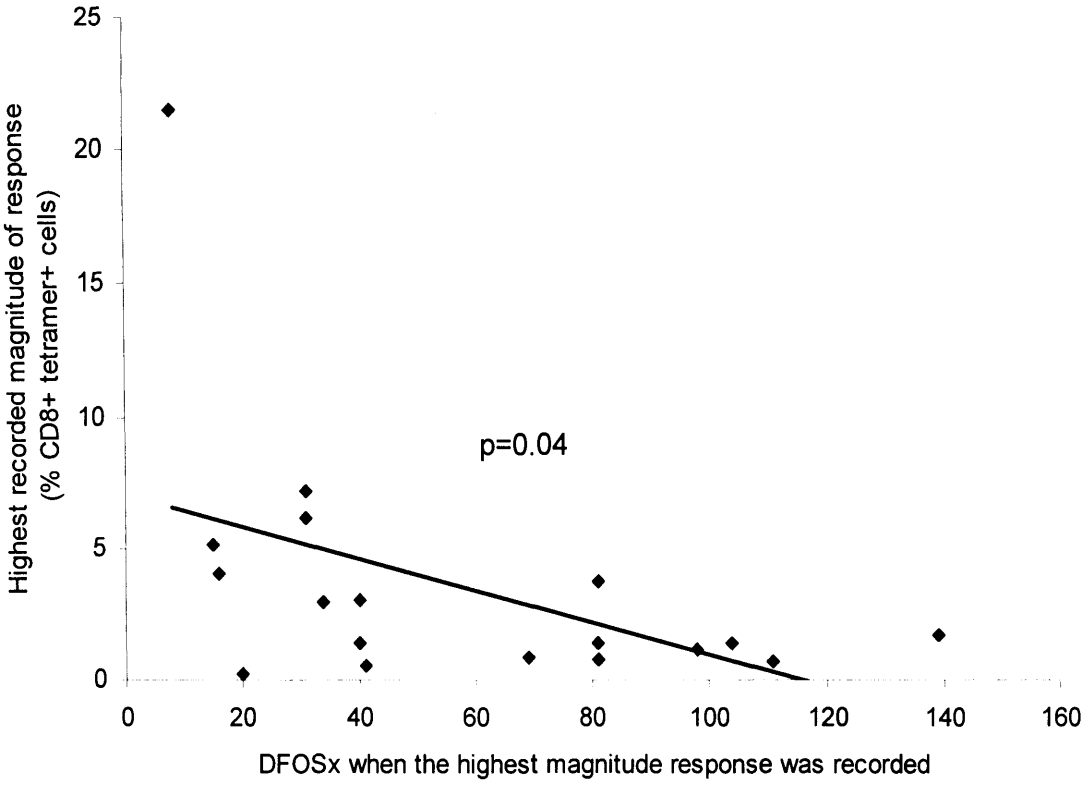


Figure 5.4. Relationship between the kinetics and magnitude of HIV epitope-specific CD8$^+$ T cell responses. The kinetics of expansion and magnitudes of epitope-specific responses in seven HIV-infected subjects were determined using tetramers as explained in the legend to Figure 5.3. For each individual epitope-specific response the DFOSx when the highest magnitude response was recorded is plotted against the highest recorded magnitude of the response. A regression line has been fitted through the data points and the p value shown (as calculated by Pearson's correlation test) indicates that there is a significant correlation between the two variables.

another sizeable response reached its highest level between 0 and 14 days after the highest recorded viral load. In all other patients, responses were slower to evolve, and at least a 14 day period was observed between the estimated peak viral load and highest recorded magnitude of the epitope-specific response. It would have been of great interest to have extended this analysis to further patients who controlled viral replication efficiently, to determine whether rapid expansion kinetics are a common feature of the primary HIV-specific $CD8^+$ T cell response in such individuals.

In summary, tetramer analysis of epitope-specific responses in seven infected individuals revealed that very high magnitude responses can be induced in primary infection, and that there are heterogeneous patterns in the dynamics of expansion of different responses both within and between different patients. A negative correlation was found between the magnitude and kinetics of expansion of the responses that were able to be studied, with faster evolving responses being of greater magnitude. A patient who controlled viral replication very efficiently was found to mount a high magnitude HIV-specific CD8 response extremely quickly.

5.3 Analysis of changes in the clonal composition of HIV epitope-specific responses over the course of infection

In section 3.7, the clonal composition of different HIV epitope-specific responses was explored. Vβ family usage by epitope-specific cells was assessed at a timepoint corresponding to the peak of each response, to compare the breadth of clonal usage in patients who established different persisting viral loads. However it was also of interest to investigate whether changes in the clonal composition of cells involved in the response to a given epitope occurred over time. To explore this, Vβ family usage by epitope-specific T cells in four patients was assessed at sequential timepoints. The four patients chosen for study established persisting viral loads in the four different viral load quartiles. PBMC cryopreserved at sequential timepoints during evolution of the response were co-stained with the relevant MHC peptide tetramer and antibodies specific for CD3, CD8 and one of a panel of antibodies specific for different TCR Vβ families, and the percentages of tetramer-stained cells using each Vβ family was determined by flow cytometry. (Figures 5.5-5.8). The timepoints analysed were selected on the basis of results from section 5.2 and were chosen so that the tetramer-positive population of cells was large enough for study (preferably >1% of $CD8^+$ T

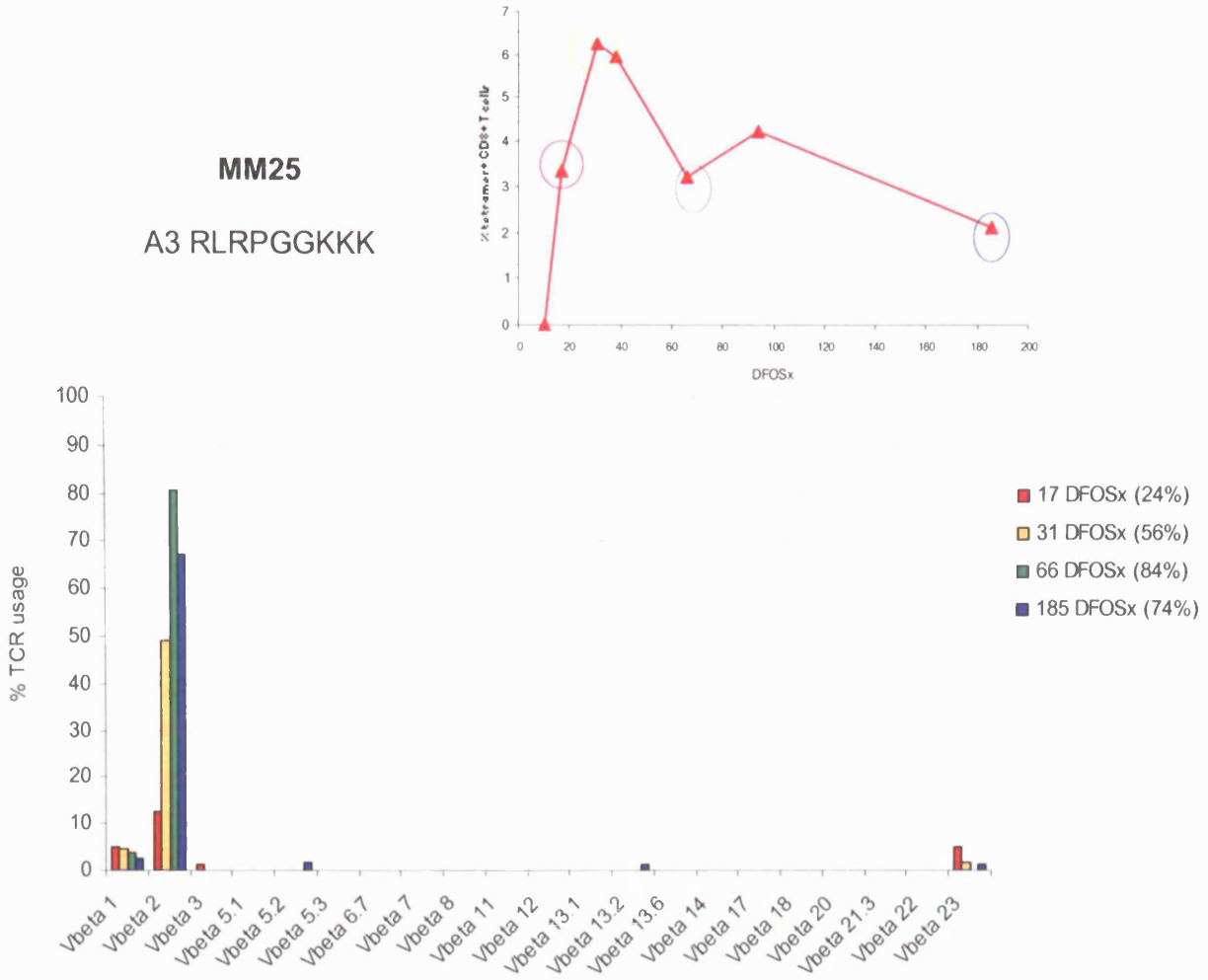


Figure 5.5. Analysis of changes in the clonal breadth of a HIV epitope-specific response over time in a patient who established a high persisting viral load. The Vβ families used by the T cell receptors of cells responding to the HIV epitope RLRPGGKKK were determined at several timepoints during infection in patient MM25. The magnitude and kinetics of expansion of the RLRPGGKKK-specific response is shown in the line graph accompanying the bar chart, with the timepoints when analysis of Vβ usage was carried out being indicated by circles. Cryopreserved PBMC were co-stained with a RLRPGGKKK tetramer, antibodies against CD3 and CD8 and an antibody specific to one of 22 different TCR Vβ families (or an isotype control antibody). The percentage of tetramer-positive cells using each Vβ family at each timepoint was determined by flow cytometry and is shown in the bar charts. Vβ antibody staining of <1% of tetramer-positive cells was regarded as background and is therefore not shown. The percentage of the entire tetramer-positive population detected by staining using the Vβ family antibodies that were available at each timepoint is indicated in brackets in the key.

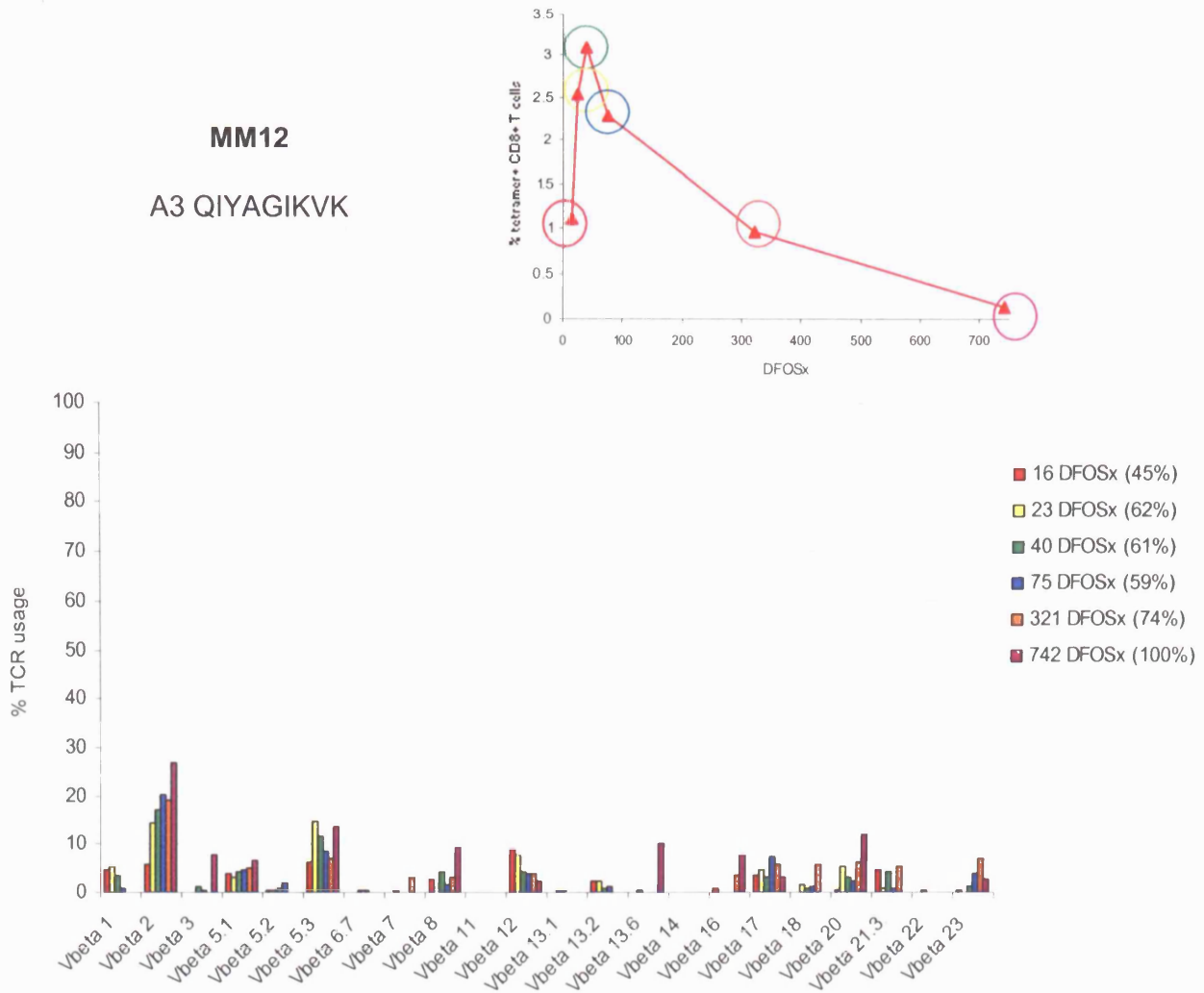
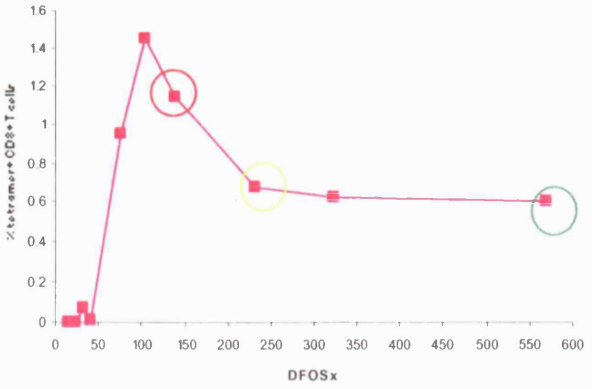
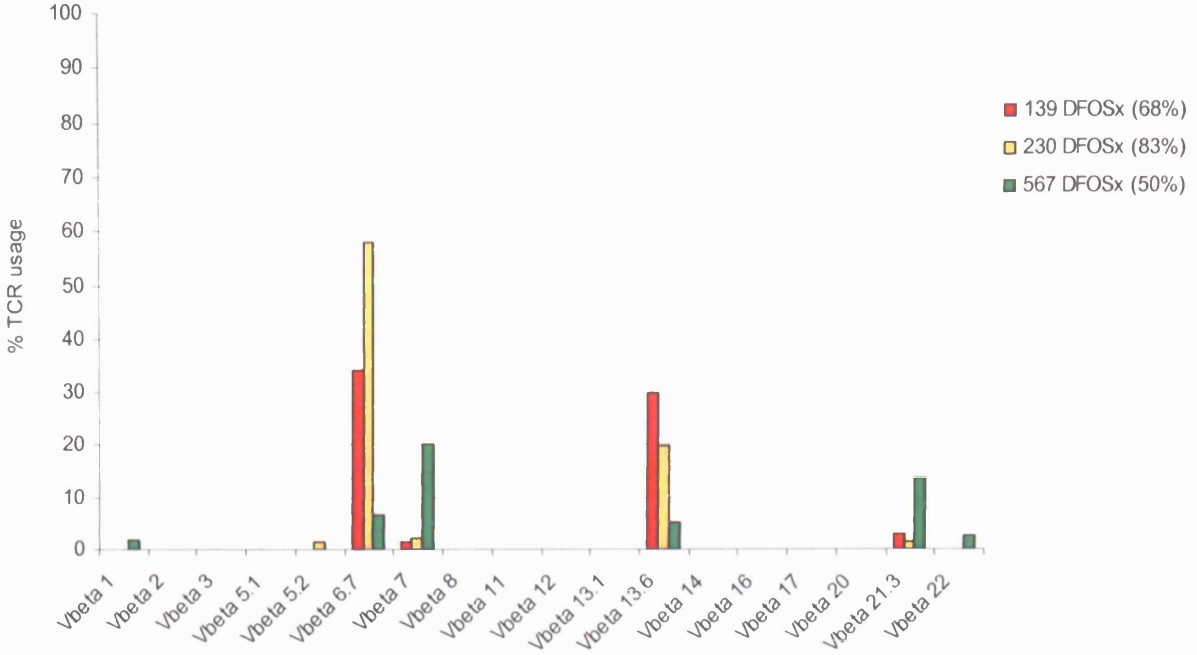


Figure 5.6. Analysis of changes in the clonal breadth of HIV epitope-specific responses over time in a patient who established an intermediate to high persisting viral load. The Vβ families used by the T cell receptors of cells responding to the HIV epitopes QIYAGIKVK and IPRRIRQGL were determined at several timepoints during infection in patient MM12. The magnitude and kinetics of expansion of each epitope-specific response is shown in the line graph accompanying each bar chart, with the timepoints when analysis of Vβ usage was carried out being indicated by circles. Cryopreserved PBMC were co-stained with an appropriate MHC class I tetramer, antibodies against CD3 and CD8 and an antibody specific to one of 22 different TCR Vβ families (or an isotype control antibody). The percentage of tetramer-positive cells using each Vβ family at each timepoint was determined by flow cytometry and is shown in the bar charts. Vβ antibody staining of <1% of tetramer-positive cells was regarded as background and is therefore not shown. The percentage of the entire tetramer-positive population detected by staining using the Vβ family antibodies that were available at each timepoint is indicated in brackets in the key.

MM12

B7 IPRRIRQGL





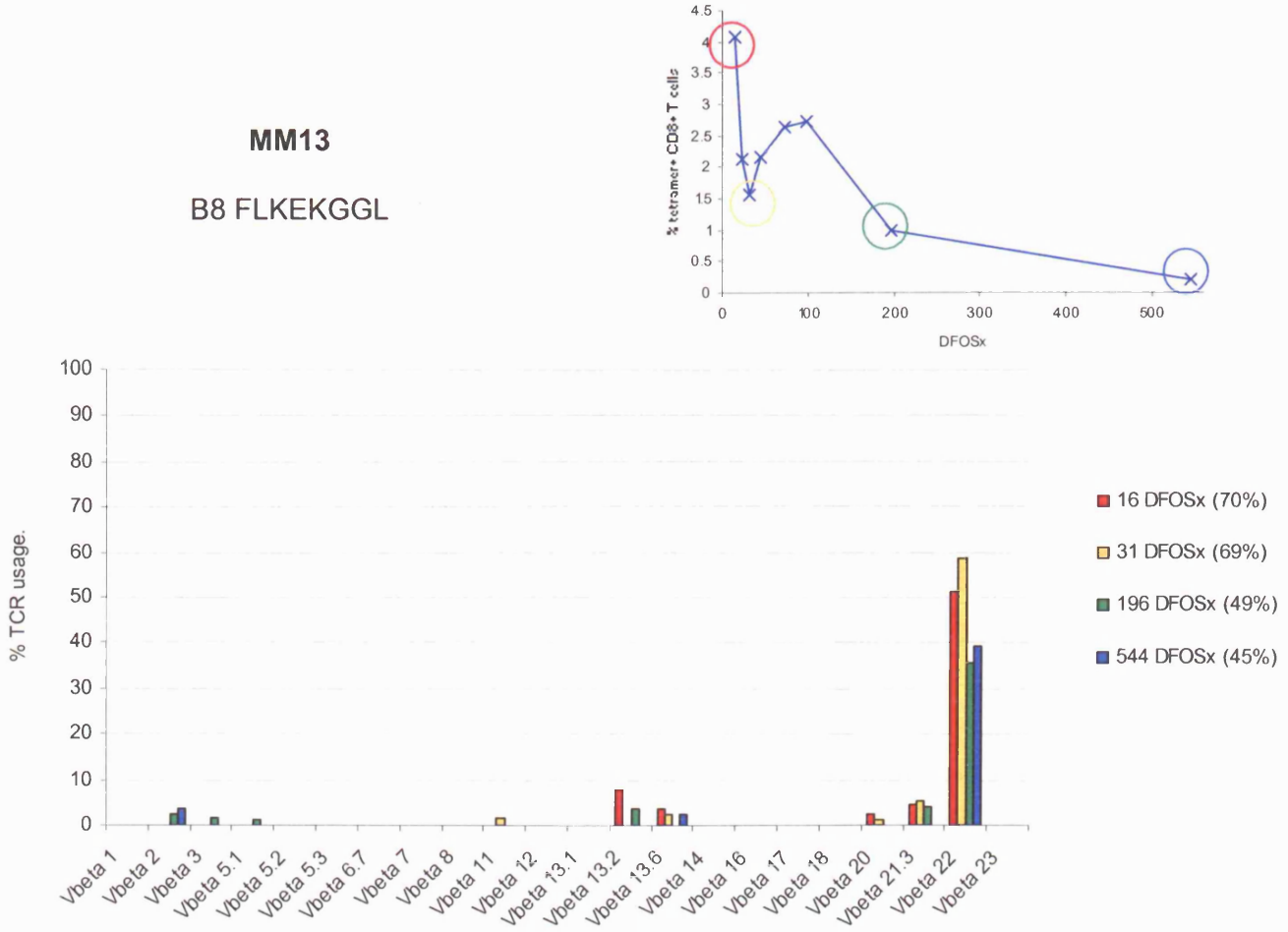
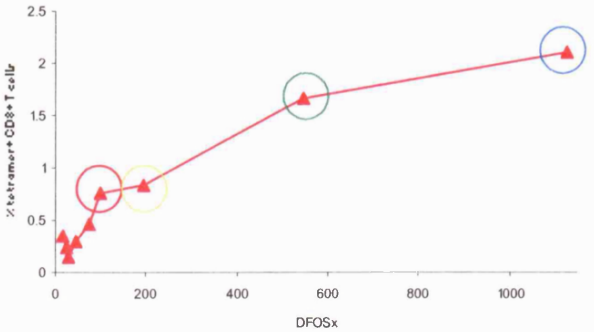
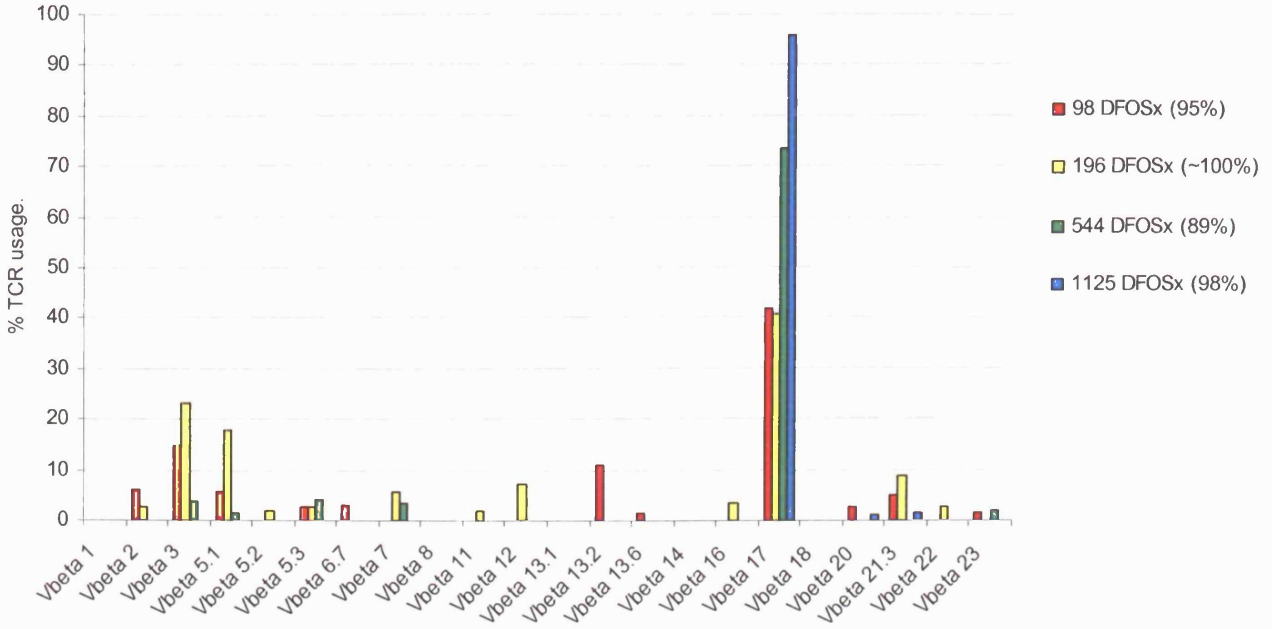


Figure 5.7. Analysis of changes in the clonal breadth of HIV epitope-specific responses over time in a patient who established an intermediate to low persisting viral load. The Vβ families used by the T cell receptors of cells responding to the HIV epitopes FLKEKGGL and KAFSPEVIPMF were determined at several timepoints during infection in patient MM13. The magnitude and kinetics of expansion of each epitope-specific response is shown in the line graph accompanying each bar chart, with the timepoints when analysis of Vβ usage was carried out being indicated by circles. Cryopreserved PBMC were co-stained with an appropriate MHC class I tetramer, antibodies against CD3 and CD8 and an antibody specific to one of 22 different TCR Vβ families (or an isotype control antibody). The percentage of tetramer-positive cells using each Vβ family at each timepoint was determined by flow cytometry and is shown in the bar charts. Vβ antibody staining of <1% of tetramer-positive cells was regarded as background and is therefore not shown. The percentage of the entire tetramer-positive population detected by staining using the Vβ family antibodies that were available at each timepoint is indicated in brackets in the key.

MM13

B57 KAFSPEVIPMF





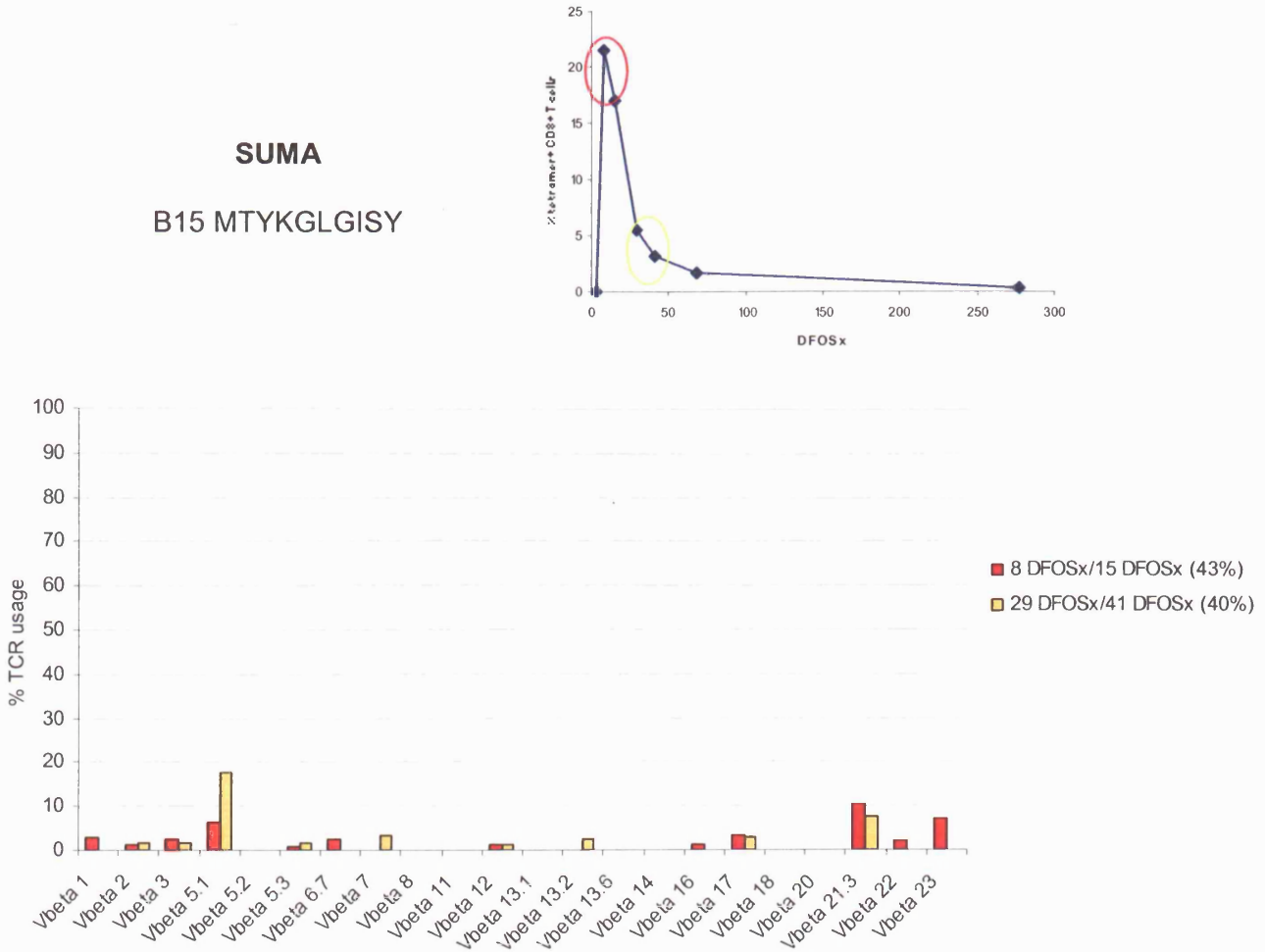
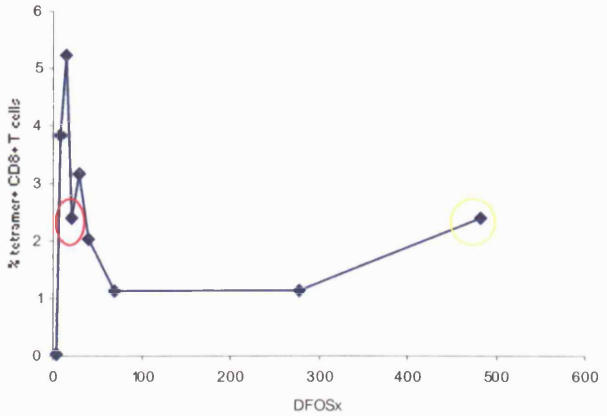
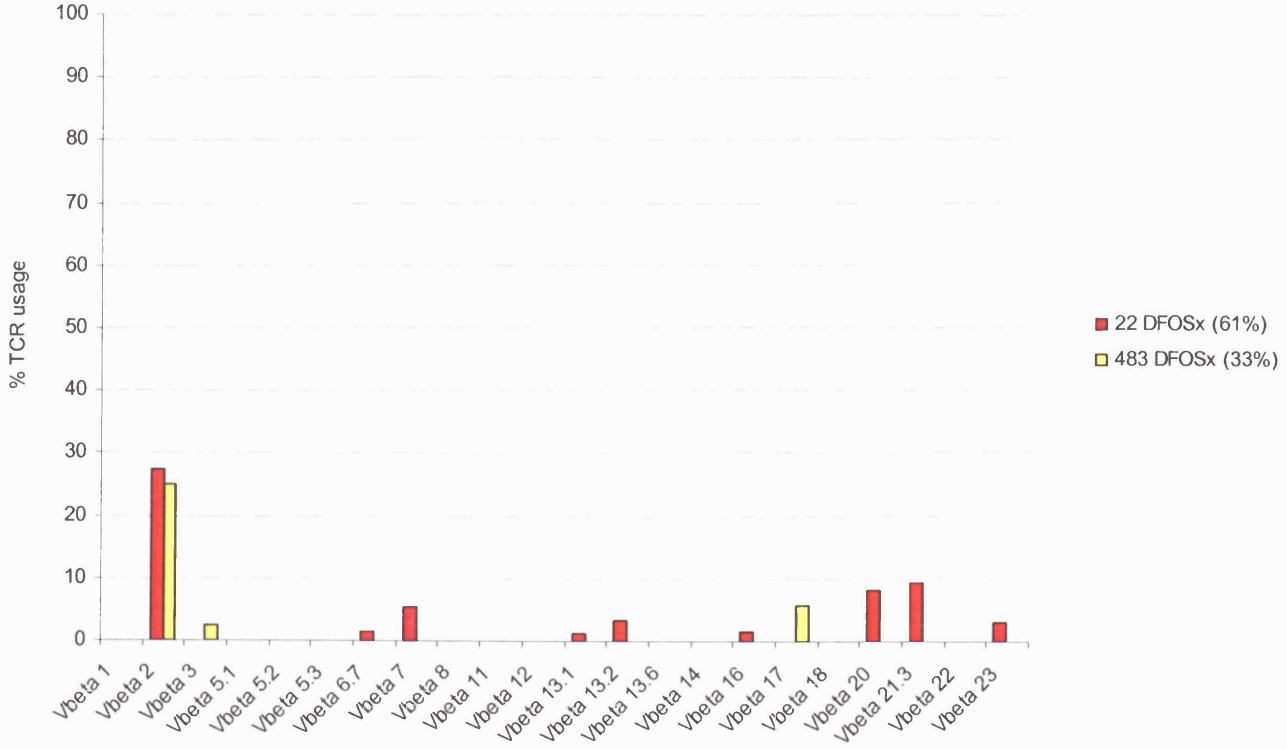


Figure 5.8. Analysis of changes in the clonal breadth of HIV epitope-specific responses over time in a patient who established a low persisting viral load. The Vβ families used by the T cell receptors of cells responding to the HIV epitopes MTYKGLGISY and ERYLKDQQL were determined at several timepoints during infection in patient SUMA. The magnitude and kinetics of expansion of each epitope-specific response is shown in the line graph accompanying each bar chart, with the timepoints when analysis of Vβ usage was carried out being indicated by circles. Cryopreserved PBMC were co-stained with an appropriate MHC class I tetramer, antibodies against CD3 and CD8 and an antibody specific to one of 22 different TCR Vβ families (or an isotype control antibody). The percentage of tetramer-positive cells using each Vβ family at each timepoint was determined by flow cytometry and is shown in the bar charts. Vβ antibody staining of <1% of tetramer-positive cells was regarded as background and is therefore not shown. The percentage of the entire tetramer-positive population detected by staining using the Vβ family antibodies that were available at each timepoint is indicated in brackets in the key.

SUMA

B14 ERYLKDQQL





cells), and where possible, corresponded to when the response was expanding, at the peak, in decline plus late into infection.

The A3 RLRPGGKKK-specific response was studied in patient MM25 (Figure 5.5). This was one of this patient's dominant responses during early infection, and expanded relatively rapidly following the peak in viral load. The clonal composition of T cells targeting this epitope was analysed at four timepoints: 17 DFOSx as the response was expanding, 31 DFOSx at the peak of the response, 66 DFOSx as the response was coming down, and at 185 DFSOx (a later timepoint). At 17 DFOSx, only a quarter of the response was detected using the available Vβ family antibodies. The epitope-specific cells detected used Vβs 1, 2 and 23 – it is likely that remaining cells also used a limited repertoire of Vβs, with the bulk of the epitope-specific T cells potentially using TCRs from just one or two unidentified families. By as early as 31 DFOSx, a marked change was observed in Vβ family use by epitope-specific T cells, with almost 50% of cells now using TCRs in the Vβ2 family. At later timepoints, there was an even greater bias towards Vβ2 usage, with over 80% of cells using this family at 66 DFOSx. At 185 DFOSx, there was more involvement of other Vβ families, but still a large proportion of the responding T cells utilised Vβ2.

The Vβ family usage by T cells involved in the response to two epitopes was followed in patient MM12 (Figure 5.6): the dominant A3 QIYAGIKVK-specific response, which expanded rapidly, peaking at 40 DFOSx; and the subdominant B7 IPRRIRQGL-specific response, which was more delayed in its expansion, peaking at 104 DFOSx. The pattern of Vβ family use by T cells responding to the two epitopes was revealed to be very different. A3 QIYAGIKVK-specific cells used a broad spectrum of Vβ families, with all except 2 of the 22 families being represented at one timepoint or another. This was quite unusual, as a broad usage of Vβ families was not found to be common during primary infection. As with the RLRPGGKKK-specific response in MM25, there was increasing usage of Vβ2 over time, although MM13's QIYAGIKVK-specific response remained heterogeneous at all timepoints studied. However, once again, due to detection of only a proportion of epitope-specific T cells by the available TCR Vβ family antibodies (particularly at earlier timepoints), it cannot be ruled out that there may have been a large expansion of cells using one particular family. By contrast, the response to the IPRRIRQGL epitope showed more restricted Vβ family usage, with the majority of epitope-specific cells using TCRs in Vβ families 6.7 and 13.6.

Again here, TCR Vβ family use was found to evolve over time, with the frequency of Vβ6.7-utilising cells increasing between 139 and 230 DFOSx, and very different Vβ families contributing to the response by 567 DFOSx.

An early (FLKEKGGEL) and a late response (KAFSPEVIPMF) were also studied in patient MM13 (Figure 5.7). The Vβ usage of FLKEKGGL-specific cells was biased towards one family, Vβ22. This was observed at each timepoint studied, from the peak of the response to late on during infection. Although the KAFSPEVIPMF-specific response was initially skewed towards the usage of one particular family (Vβ17), there was substantial involvement of other families at the earlier timepoints. However, as the total epitope-specific response increased in magnitude, there was increasing usage of Vβ17, with over 90% of tetramer positive cells using this family by 1125 DFOSx. For this particular response, the patient therefore started off with a broader repertoire, which was narrowed down over time.

In patient SUMA, the dominant MTKGLGISY-specific response peaked a week prior to the less dominant ERYLKDQQL-specific response. Both responses were only studied at two timepoints due to sample shortage, one earlier and one later timepoint during the course of infection (Figure 5.8). Cells targeting the MTKGLGISY epitope utilised a number of Vβ families, each family accounting for only a small proportion of cells at both timepoints, and no major changes were seen from one timepoint to the next. However, since under half of the total epitope-specific response was detected using the available Vβ family antibodies at both timepoints, it cannot be ruled out the remainder of the undetected response may have been quite biased towards one particular Vβ family. ERYLKDQQL-specific cells also used a substantial number of Vβ families (9 out of 22) at an subacute/early timepoint (22 DFOSx), with almost 30% using Vβ2. However by 483 DFOSx, the relatively broad repertoire at an early timepoint (22 DFOSx) had now narrowed down to usage of only three families, with Vβ2 remaining prominent. Again, however, the available panel of Vβ family-specific antibodies did not stain all epitope-specific T cells, hence a substantial proportion of epitope-specific T cells may have utilised one or more unidentified Vβ families.

In summary, the patterns of Vβ family utilisation by TCRs of different epitope-specific cells were quite variable both within patients and between different patients. In some cases the initial responses were restricted, with bias towards utilisation of one particular Vβ family, the degree of which may or may not have increased over time. In other cases heterogeneous responses initially

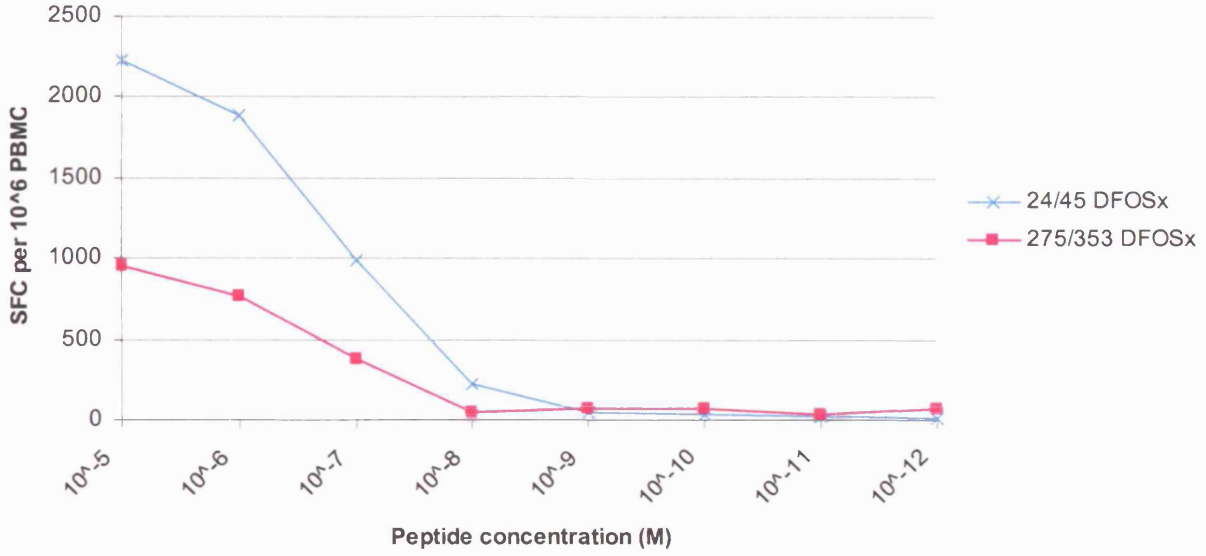
observed, which either remained so at all timepoints studied, or became more restricted over time. However, as mentioned several times, these results should be interpreted with caution since, due to the failure to detect all cells specific for certain epitopes using the available panel of Vβ family antibodies, important features of certain responses may have been missed.

5.4 Comparison of the relative avidity of HIV epitope-specific T cells at different stages of infection

Results from section 5.3 suggested that the repertoire of T cells involved in the response to certain epitopes evolved over time. In some cases there was evidence of selection for T cells using TCR(s) from a particular Vβ family within the epitope-specific T cell population over time. This leads to the question of why such selective changes took place. One possibility is that as TCR use may affect the avidity of interaction between $CD8^+$ T cells and the APC or target cell, and so T cells using particular TCRs may have come to dominate the epitope-specific T cell population over time because they interacted with epitope-bearing cells with high avidity. Other possible reasons as to why selection for certain T cells may have occurred over time are considered in the discussion. Here the temporal relationship between TCR Vβ family usage and the avidity of the response was studied.

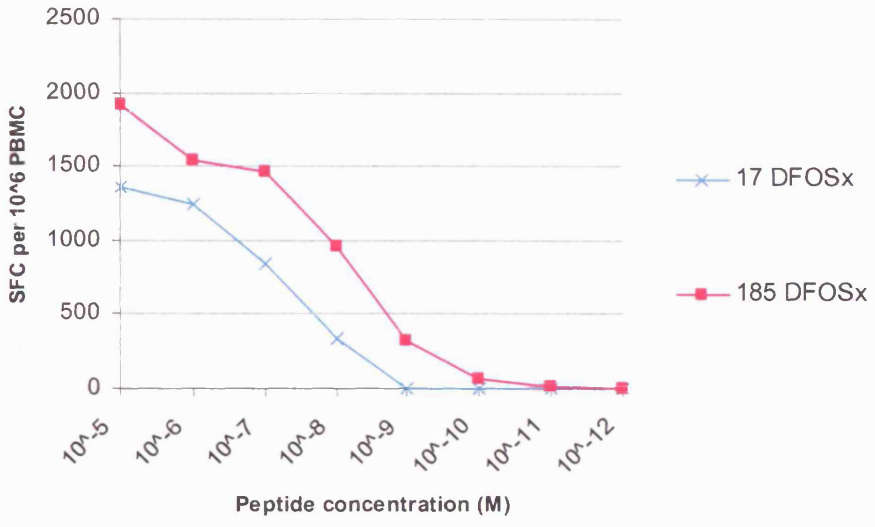
To address this, the relative avidities of the epitope-specific T cell populations present at early and late timepoints were compared to see if, coincident with the selection for epitope-specific cells bearing TCR(s) in a certain Vβ family, there were changes in the avidity of the response and the T cells from the later timepoint were of higher avidity. Possible changes in the avidity of the T cell response were studied for two epitopes, one where epitope-specific cells exhibited stable Vβ family use (FLKEKGGL-specific cells in MM13) (Figure 5.9) and one where there was selection for preferential usage of one particular Vβ family over time (RLRPGGKKK in MM25) (Figure 5.10). As described in section 4.2, the relative avidities of T cell responses were measured by determining the concentration of the epitope peptide required to stimulate half of the maximum response in an IFN-γ ELISPOT assay.

The avidity of the T cell response to the FLKEKGGL epitope in patient MM13 had already been determined using cells cryopreserved at a late timepoint (cells combined from 275 DFOSx and 353 DFOSx). T cells from this stage of infection required a peptide concentration of 10^{-7}M to stimulate half of the maximum specific IFN-γ response (Figure 4.3a). Here, the avidity of T cells



Epitope peptide	Concentration of peptide required to stimulate half maximum response	
	Early timepoint	Late timepoint
FLKEKGGL	10^{-7}M	10^{-7}M

Figure 5.9. Comparison of the relative avidity of the T cell response made to the B8 FLKEKGGL epitope by patient MM13 at an early and a late timepoint during infection. The response of PBMCs cryopreserved from patient MM13 at an early (cells combined from 24 DFOSx and 45 DFOSx) and a late (cells combined from 275 DFOSx and 353 DFOSx) timepoint to a peptide corresponding to the FLKEKGGL epitope at a range of dilutions from 10^{-5}M to 10^{-12}M, was determined by IFN-γ ELISPOT assay. Results are expressed as specific SFC per 10^6 PBMC (i.e. after deduction of the background level of IFN-γ production). The concentration of peptide required to stimulate half the maximum response (defined as that observed when cells were stimulated with peptide at 10^{-5}M) was calculated as a measure of the relative avidity of the T cell response made to the epitope at each timepoint.



Epitope peptide	Concentration of peptide required to stimulate half maximum response	
	Early timepoint	Late timepoint
RLRPGGKKK	10^{-7}M	10^{-9}M

Figure 5.10. Comparison of the relative avidity of the T cell response made to the A3 RLRPGGKKK epitope by patient MM25 at an early and a late timepoint. The response of PBMCs cryopreserved from patient MM25 at an early (17 DFOSx) and a late (185 DFOSx) timepoint to a peptide corresponding to the RLRPGGKKK epitope at a range of dilutions from 10^{-5}M to 10^{-12}M, was determined by IFN-γ ELISPOT assay. Results are expressed as specific SFC per 10^6 PBMC (i.e. after deduction of the background level of IFN-γ production). The concentration of peptide required to stimulate half the maximum response (defined as that observed when cells were stimulated with peptide at 10^{-5}M) was calculated as a measure of the relative avidity of the T cell response made to the epitope at each timepoint.

targeting this epitope was also determined at an early timepoint (using cells combined from 24 DFOSx and 45 DFOSx). The peptide dose-response curves of cells at both stages of infection are shown in Figure 5.9. This illustrates that although the magnitude of the response at the earlier timepoint was more than twice that at the later timepoint, the peptide concentration required to stimulate half the maximum response was still 10^{-7}M. Therefore for this epitope-specific response, there was no change in the avidity of the response with time.

The avidities of T cells responding to the RLRPGGKKK epitope in patient MM25 were also measured at earlier (17 DFOSx) and later (185 DFOSx) stages of infection. In Figure 5.10 it can be seen that a lower concentration of peptide was required to stimulate half of the maximum level of response when cells from the later timepoint were used in the assay compared to cells from the earlier timepoint. The relative avidity of the response was 10^{-7}M at the earlier timepoint, and 10^{-9}M at the later timepoint. This may have been due to selection of T cells with higher affinity TCR(s), which could in turn be related to the skewing of the T cell repertoire towards utilisation of one dominant Vβ family (Vβ2), as seen in Figure 5.5. Experiments that could have been carried out to address the relationship between TCR Vβ family usage and the avidity of responses in more depth are considered in the discussion.

5.5 Phenotypic characterisation of HIV-specific CD8$^+$ T cells

In addition to repertoire-related aspects of an epitope-specific CD8$^+$ T cell response, the phenotype of the cells can also be studied using tetramers to find out more about the qualitative aspects of the response. It has been suggested that there may be defects in the maturation and/or functional properties of HIV-specific T cells (Appay *et al.*, 2000; Appay *et al.*, 2002; Champagne *et al.*, 2001; Goepfert *et al.*, 2000; Kostense *et al.*, 2001; Petrovas *et al.*, 2004; Shankar *et al.*, 2000; van Baarle *et al.*, 2002a), for despite the induction of high magnitude virus-specific CD8$^+$ T cell responses during early HIV-1 infection, the virus is still able to replicate at high levels. In this section, the phenotype of epitope-specific T cells was studied over the course of infection to see if any defects in T cell maturation could be detected, and if so how early in infection they were acquired (other studies have predominantly focused upon chronic infection (Appay *et al.*, 2000; Champagne *et al.*, 2001; Goepfert *et al.*, 2000; Kostense *et al.*, 2001; Shankar *et al.*, 2000)). Patients representative of the four viral load quartiles were studied (MM25, MM12, MM13 and SUMA, representing patients who established high

down to low persisting viral loads) to see if there were any differences in the phenotype of the HIV-specific T cells in these individuals (which could be either a consequence or a cause of the differing efficiency with which early viral replication was controlled). Where possible, the phenotypic analysis was carried out for T cells specific for both an immunodominant and a subdominant epitope within each patient's early response to see if there were any qualitative differences in these quantitatively different responses. In patient MM25 the dominant RLRPGGKKK-specific response was monitored over time; in patient MM12, both early expanding dominant QIYAGIKVK-specific T cells and later emerging subdominant IPRRIRQGL-specific T cells were studied; in patient MM13, the early dominant response towards FLKEKGGL and the later response to KAFSPEVIPMF were both phenotypically characterised, and in patient SUMA, the dominant MTKGLGISY-specific response, and the somewhat less dominant ERYLKDQQL-specific response were followed.

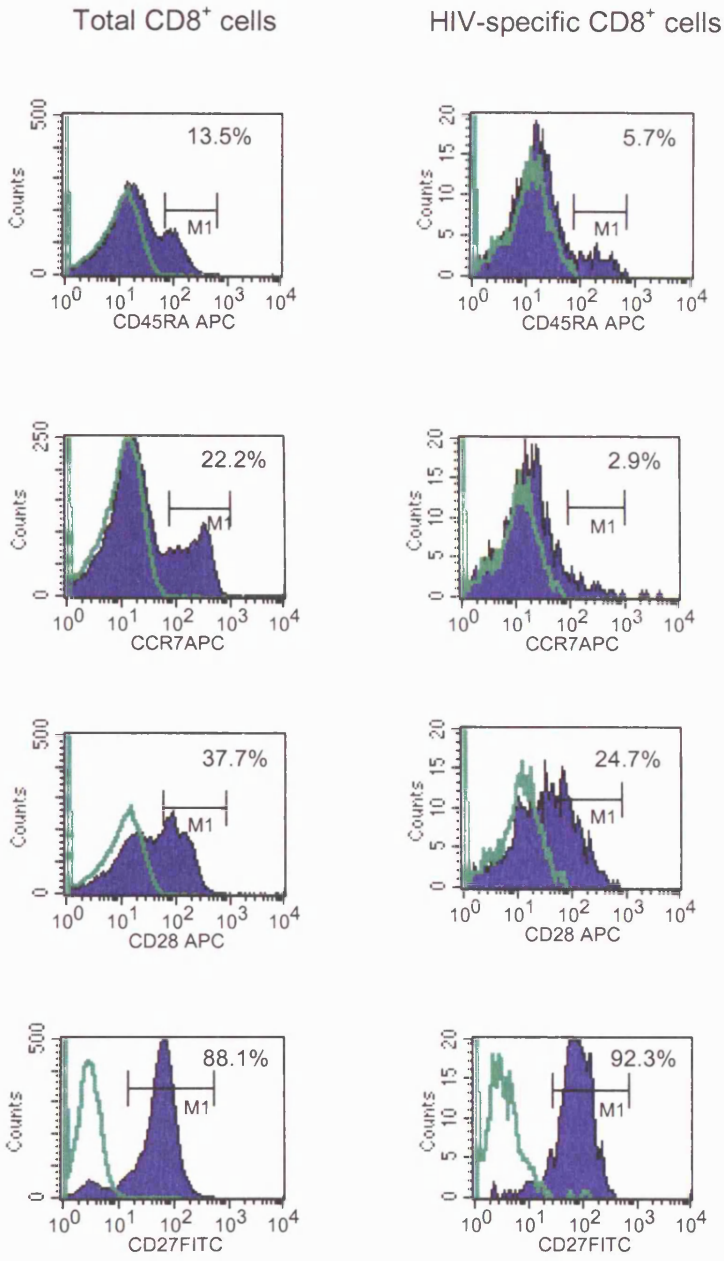
5.5.1 Changes in differentiation status undergone by HIV-specific $CD8^+$ T cells over the course of infection

The expression of the markers CCR7, CD27, CD28 and CD45RA can be used to determine the differentiation/maturation status of $CD8^+$ T cells (Beverley, 1987; Hamann *et al.*, 1997; Sallusto *et al.*, 1999). Naïve $CD8^+$ T cells are characterised by a $CCR7^+$ $CD27^+$ $CD28^+$ $CD45RA^+$ phenotype. Following antigenic stimulation, antigen-experienced cells mature into memory and effector T cells: the cells start to express the RO rather than the RA isoform of the CD45 molecule and sequentially down regulate CCR7, CD28 and CD27. Memory cells are often defined by the expression of the RO isoform of CD45, with the central memory subset expressing CCR7 and effector memory subset lacking CCR7 expression (Sallusto *et al.*, 1999). Fully differentiated effector T cells are thought to have a $CCR7^-$ $CD27^-$ $CD28^-$ phenotype and may also re-express CD45RA (Appay *et al.*, 2002; Champagne *et al.*, 2001; Dunne, 2002; Hamann, 1999; Wills, 2002). Studies of chronically-infected patients have suggested that HIV-specific $CD8^+$ T cells may be unable to differentiate into fully mature effector cells, and are arrested at a pre-terminally differentiated stage of maturation where they are predominantly of the phenotype $CCR7^-$ $CD27^+$ $CD28^-$ $CD45RO^+$ (Appay *et al.*, 2000; Champagne *et al.*, 2001). To determine whether cells present in the acute phase of infection also possess this 'immature' phenotype, and if not, to assess the kinetics with which this

phenotype may be acquired, the expression of CCR7, CD28, CD27 and CD45RA was characterised on tetramer-positive cells of different specificities during different phases of the expansion and decline of epitope-specific responses. PBMC cryopreserved at various timepoints throughout the course of infection were thawed and stained with a HIV tetramer (or pentamer), an anti-CD8 antibody and antibodies specific for CCR7, CD27, CD28 and CD45RA. The expression of these fluorescent markers on tetramer-labelled cells was then determined by four-colour flow cytometry. Since the use of four-colour flow cytometry enabled $CD8^+$ tetramer-positive cells to be simultaneously stained with antibodies specific for two other markers, co-staining with combinations of antibodies of potential interest was carried out.

Figure 5.11 shows the expression of CD45RA, CCR7, CD27 and CD28 on the total CD8 lymphocyte population and on the HIV QIYAGIKVK-specific $CD8^+$ cells in patient MM13 at 40 DFOSx (the QIYAGIKVK-specific population representing 3-4% of the total CD8 population). $CD45RA^+$ and $CD45RA^-$ populations of T cells are evident within the total CD8 pool, with the balance between the populations being heavily skewed towards $CD45RA^-$ cells. Notably, the majority of HIV $tetramer^+$ cells are also $CD45RA^-$: the high percentage of HIV-specific $CD8^+$ T cells present in the blood at this timepoint is thus likely responsible for biasing the composition of the total CD8 pool towards $CD45RA^-$ cells. Likewise there are also more $CCR7^-$ than $CCR7^+$ cells in the total CD8 T cell pool, and the majority of HIV $tetramer^+$ cells have a $CCR7^-$ phenotype. Both $CD28^+$ and $CD28^-$ populations are evident within the total CD8 pool. The HIV $tetramer^+$ cells also include both $CD28^+$ and $CD28^-$ subsets, although a higher proportion of cells in the overall $CD8^+$ T cell pool express CD28 than is observed in the HIV $tetramer^+$ population. Only a low percentage of all CD8 T cells are $CD27^-$; and the majority of HIV $tetramer^+$ $CD8^+$ T cells are also $CD27^+$. The HIV-specific population contains fewer $CD27^-$ $CD28^-$ cells than the total CD8 population, which suggests that although this subject has a reasonable proportion of $CD8^+$ cells with characteristics of more advanced stages of differentiation, their HIV-specific cells are not part of this subset of cells and do not have the phenotypic characteristics of fully differentiated effector cells. Of the $CD45RA^+$ cells, similar proportions in the HIV-specific population and total CD8 population are also $CD28^-$, the phenotype associated with the CD45RA revertant population. Compared to the total CD8 population, the vast majority of HIV-specific cells are $CCR7^-$ $CD27^+$, consistent with them having seen antigen but being at a relatively early

(A)


Total CD8+ cells
HIV-specific CD8+ cells
13.5%
5.7%
M1
Counts
CD45RA APC
22.2%
2.9%
CCR7APC
37.7%
24.7%
CD28 APC
88.1%
92.3%
CD27FITC

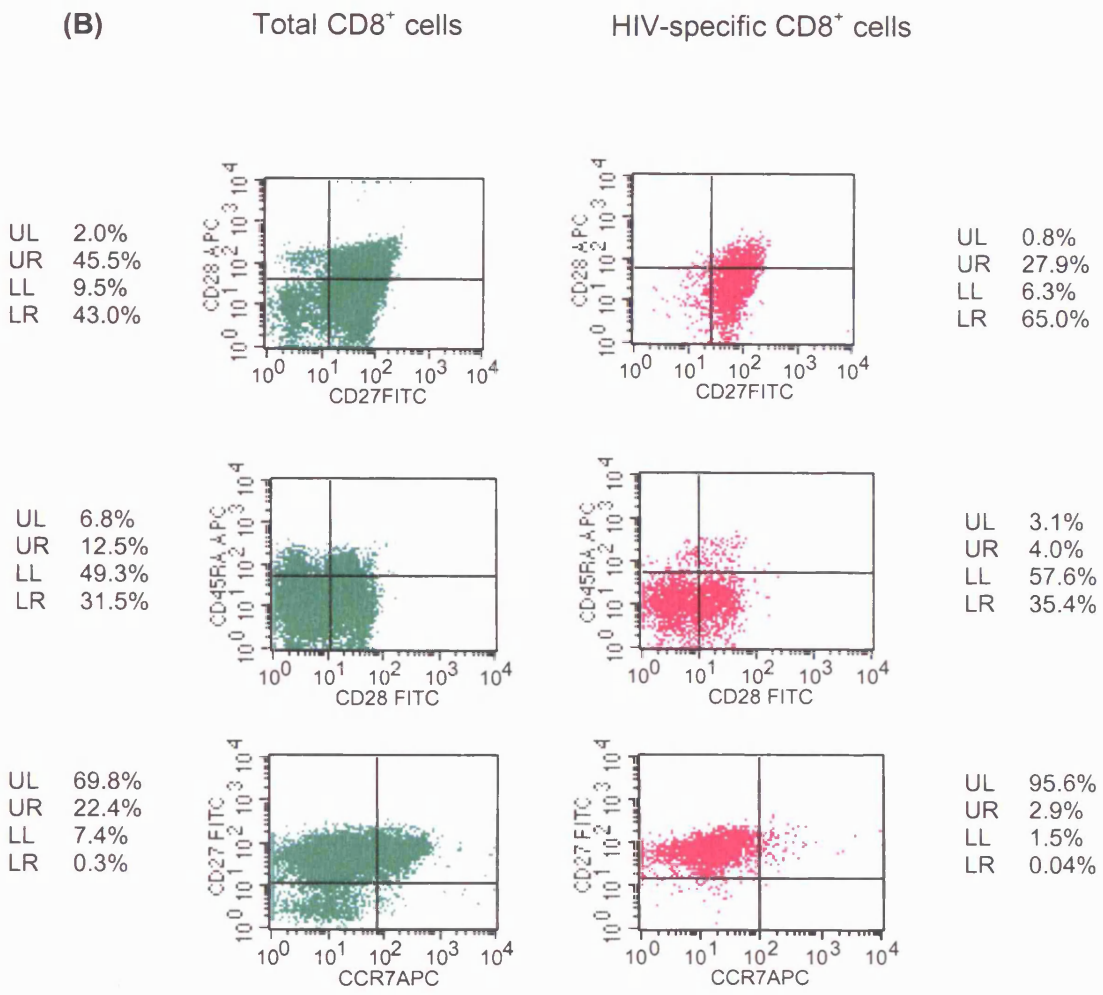


Figure 5.11. CD45RA, CCR7, CD28 and CD27 and expression on total CD8$^+$ cells and HIV-specific CD8$^+$ cells in patient MM12. Cells cryopreserved from patient MM12 at 40 DFOSx were co-stained with an A3 QIYAGIKVK tetramer and antibodies specific for CD8 and either CD45RA, CCR7, CD28 or CD27. Plots on the left are gated on the CD8$^+$ lymphocyte population, and those on the right on the CD8$^+$ tetramer$^+$ population. In (A) the proportion of cells in the total CD8$^+$ lymphocyte population or the CD8$^+$ tetramer$^+$ population expressing each of the markers (blue histograms) at levels above the background staining of cells incubated with appropriate isotype control antibodies (green histograms) is indicated in the M1 region of each plot. In (B) co-expression of CD27 and CD28, CD28 and CD45RA, and CCR7 and CD27 on the total CD8$^+$ and HIV-specific CD8$^+$ populations are shown, and the percentage of cells in each quadrant expressing the indicated combination of markers is given.

stage of differentiation, whereas the total CD8 population is more heterogeneous in the expression of the two markers.

The expression of CD45RA, CCR7, CD27 and CD28 on CD8 cells specific for the A3 QIYAGIKVK epitope in patient MM12 at different stages of the evolution of the response (16 DFOSx and 23 DFOSx, two timepoints when the response was expanding; 40 DFOSx at the peak of the response; then 75 DFOSx, 321 DFOSx and 742 DFOSx, as the response was declining) is shown in Figure 5.12. All histogram plots were gated on the $CD8^+$ $tetramer^+$ population of cells indicated and the percentage of $CD8^+$ $tetramer^+$ cells expressing each marker is given. The majority of tetramer-positive cells were $CD45RA^-$ during early infection, but at later timepoints (321 and 742 DFOSx), there was a proportion of cells which re-expressed this marker. CCR7 was not found to be expressed on tetramer-positive cells at any timepoint (not shown). CD27 was expressed by over 90% of tetramer-positive cells at the timepoints when the response expanded and peaked; thereafter, the percentage of $CD27^+$ tetramer positive cells was slightly lower, although CD27 expression was still found to be expressed on greater than 80% of tetramer-positive cells even at late timepoints. CD28 was expressed by a moderate percentage of the tetramer-positive population at the first timepoint analysed (26.7% at 16 DFOSx), but this percentage became gradually smaller over time, with 15% of tetramer-positive cells expressing CD28 by 742 DFOSx. Overall, from the earliest timepoint, the cells showed characteristics of having seen antigen ($CCR7^-$ $CD45RA^-$) and pre-terminal differentiation (retaining CD27 and to a lesser extent, CD28 expression). A proportion of cells at the later timepoints had some characteristics of late differentiated cells (re-expression of CD45RA).

The expression of these markers of cellular differentiation was determined in a similar manner over time for HIV-specific cells targeting other epitopes in other patients. The results of this analysis are summarised in Table 5.1, where the relative levels of expression of the different markers are represented by symbols, as explained in the figure legend.

The overall trend was for all tetramer-positive cells to be CD45RA negative at early timepoints for all epitope-specific responses studied, consistent with them having been antigen-primed; but at later stages of infection, there was evidence of some cells re-expressing CD45RA again. There was a universal lack of CCR7 expression on HIV tetramer-positive cells. CD27 was typically expressed on a high percentage of tetramer-positive cells. A slight decrease in

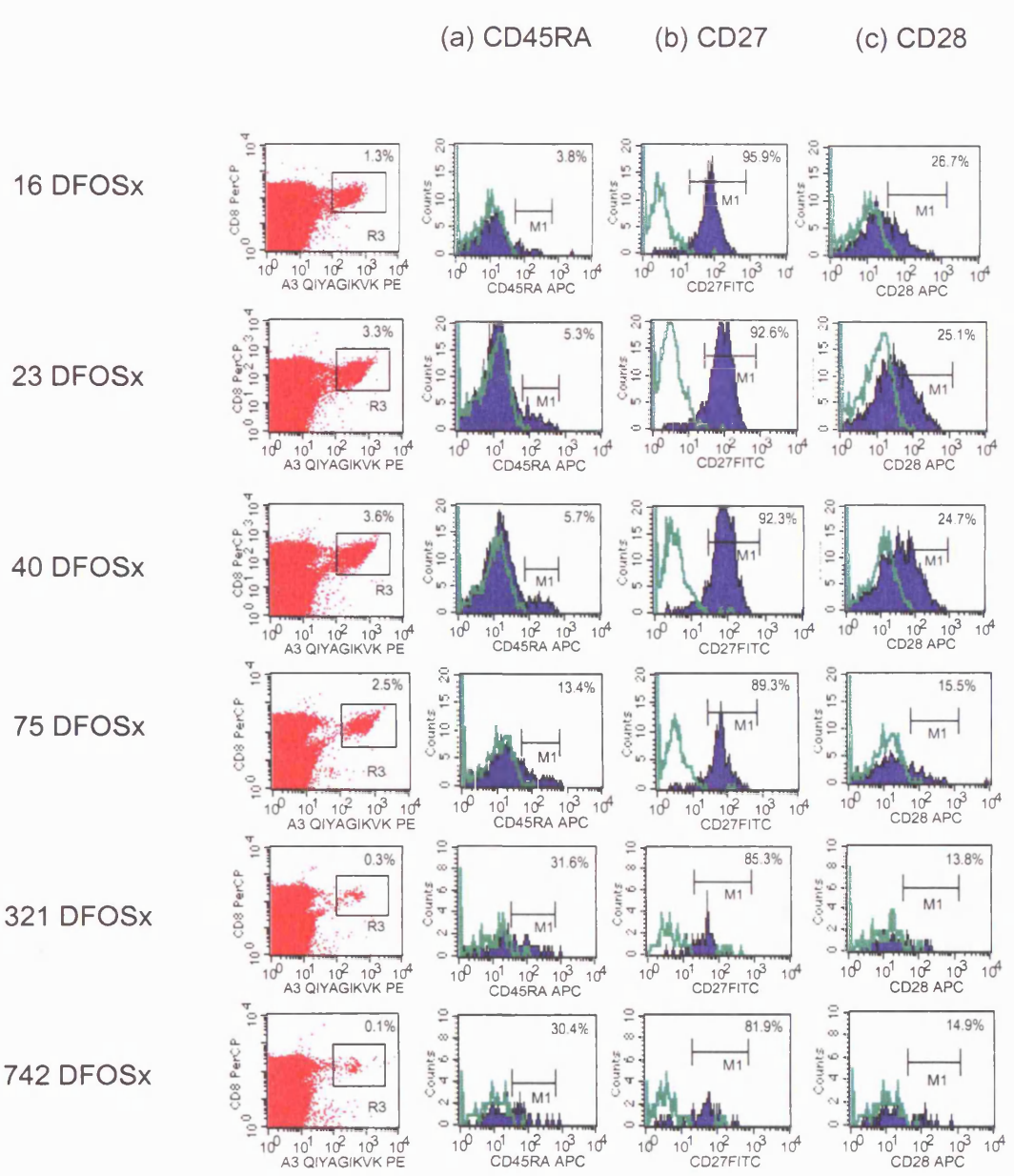


Figure 5.12. Expression of CD45RA, CD27 and CD28 on A3 QIYAGIKVK-specific CD8$^+$ cells over the course of HIV infection. Cells cryopreserved from patient MM12 at 16, 23, 40, 75, 321 and 742 DFOSx were co-stained with an A3 QIYAGIKVK-PE tetramer and anti-CD8 antibody and (a) CD45RA-APC antibody, (b) CD27-FITC antibody or (c) CD28-APC antibody. Each histogram plot is gated on the CD8$^+$ tetramer$^+$ population shown in the R3 region in the corresponding dot plot on the left. The blue histogram plot at each timepoint shows the levels of expression of the marker on the tetramer positive population above background staining of the cells with an appropriate isotype control antibody (green line). The percentages of CD8$^+$ tetramer$^+$ cells (in the R3 region) at each timepoint are given, as are the percentages of CD8$^+$ tetramer$^+$ cells expressing CD45RA, CD27 or CD28 (in the M1 region).

Patient	Specificity of CD8$^+$ T cells	Timepoint at which cells were taken for phenotyping (DFOSx)	Phenotype of HIV epitope-specific cells at indicated timepoint			
			CD45RA	CCR7	CD27	CD28
MM25	A3 RLRPGGKKK	17	-	-	+++++	+
		31	-	-	+++++	-
		66	-	-	+++++	±
		185	-	-	+++++	±
MM12	A3 QIYAGIKVK	16	-	-	+++++	+
		23	-	-	+++++	+
		40	-	-	+++++	+
		75	±	-	++++	±
	B7 IPRRIRQGL	321	+	-	++++	±
		742	+	-	++++	±
		139	+	-	++++	+
		230	+	-	+++	-
		567	+	-	+++	-
MM13	B8 FLKEKGGL	16	-	-	+++++	+
		31	±	-	+++++	±
		196	+	-	++++	±
		544	+	-	++++	+
	B57 KAFSPEVIPMF	98	±	-	+++	±
		196	±	-	++++	±
		544	-	-	++++	±
SUMA	B15 MTKGLGISY	8/15	-	-	+++++	±
		29/41	-	-	+++++	±
	B14 ERYLKDQQL	22	-	-	+++++	+
		483	±	-	++	-

Symbols represent percentage of CD8$^+$ tetramer$^+$ cells expressing a given marker at the indicated timepoint:

- 0% - 9% ± 10% - 19% + 20% - 39%
++ 40% - 59% +++ 60% - 79%
++++ 80% - 89% +++++ 90% - 100%

Table 5.1. Expression of CD45RA, CCR7, CD27 and CD28 on HIV-specific CD8$^+$ T cells over the course of infection. PBMC cryopreserved from patients MM25, MM12, MM13 and SUMA at different stages of infection were stained with the indicated tetramers (or pentamers) to identify HIV-specific CD8$^+$ T cells responding to one or two different viral epitopes, and were co-stained with fluorescently labelled antibodies specific for CD8 and CD45RA, CCR7, CD27 or CD28 to allow assessment of the phenotype of epitope-specific T cells. The percentage of CD8$^+$ tetramer$^+$ cells expressing each marker was assessed by flow cytometry, and is represented in the table by + and – signs as explained in the key.

the percentage of $CD27^+$ $tetramer^+$ cells was often observed after the peak of the response, but this marker was still expressed on the majority of cells at all timepoints studied. By comparison, CD28 was found to be expressed on a much lower percentage of cells across all timepoints. In some cases, little change was observed in the expression of this marker during different phases of the response (for example, for FLKEKGGL-specific cells in MM13), but in other cases, a decrease in the percentage of $CD28^+$ cells was observed at timepoints when the response was in decline (for example the IPRRIRQGL-specific response in MM12).

Looking at all the markers together, HIV-specific T cells did not exhibit what has been defined as a fully differentiated effector cell phenotype, i.e. $CCR7^-$ $CD28^-$ $CD27^-$ $CD45RA^+$ at any of the timepoints studied, suggesting that HIV-specific cells fail to acquire the phenotype which is associated with full effector function at any stage in infection. HIV-specific cells instead had a phenotype that was more consistent with a pre-terminally differentiated stage, retaining CD27 expression throughout infection, although at later, chronic timepoints, they did show some features of late differentiated cells (such as re-expressing CD45RA and having downregulated CD28 expression). These results indicate that defects in the maturation/differentiation of HIV-specific cells are present from acute/early infection onwards. This was the case for all patients studied, even a patient who contained viral replication well (patient SUMA). This is discussed in more depth at the end of the chapter.

5.5.2. Expression of cytokine receptors on HIV-specific $CD8^+$ T cells

IL-2 (Mier & Gallo, 1980) and IL-7 (Chatterjee *et al.*, 1994; Chazen *et al.*, 1989; Welch *et al.*, 1989) are cytokines which both have roles in stimulating T cell proliferation and maintenance. It has been suggested that altered responses to such cytokines may be among the factors that contribute to abnormalities in the HIV-specific $CD8^+$ T cell response, and that this may be associated with altered expression of the receptors for these cytokines (Mier & Gallo, 1980; Vingerhoets *et al.*, 1998). To investigate this possibility, HIV-specific cells were stained with antibodies specific for the IL-7R and CD25. CD25 is the high affinity IL-2R α chain, expression of which can also be used as a marker for T cell activation.

The expression of IL7-R and CD25 were initially compared on total CD8 cells and HIV QIYAGIKVK-specific cells at 40 DFOSx in patient MM12 (Figure 5.13). By contrast with the total CD8 cell population (29.7% of which were IL-

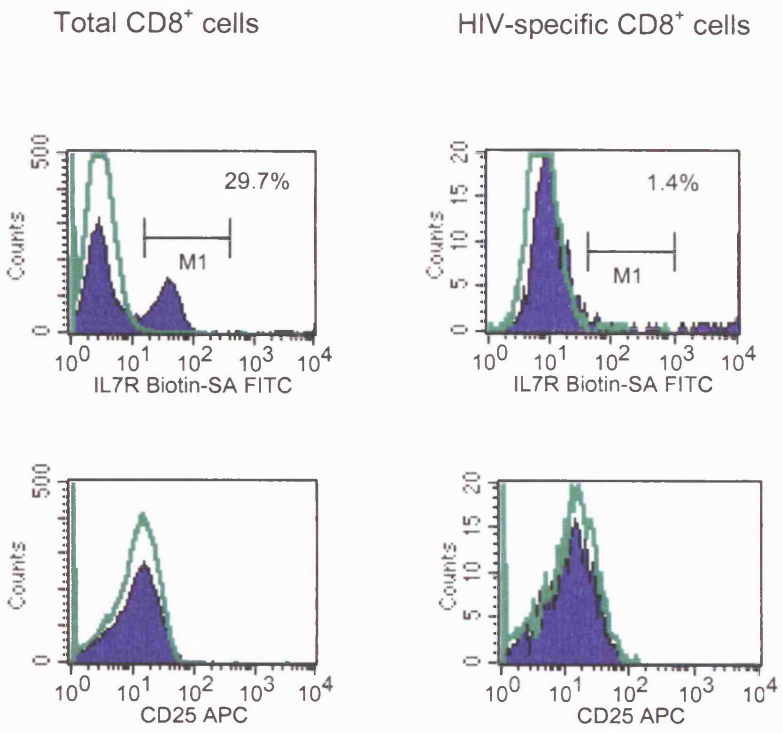


Figure 5.13. IL-7R and CD25 expression on total CD8$^+$ cells and HIV-specific CD8$^+$ cells from patient MM12. Cells cryopreserved from patient MM12 at 40 DFOSx were co-stained with an A3 QIYAGIKVK tetramer and antibodies specific for CD8 and either the IL-7R or CD25. The proportion of cells in the total CD8$^+$ lymphocyte population or the CD8$^+$ tetramer$^+$ population expressing each of the markers (blue histograms) at levels above the background staining of cells incubated with appropriate isotype control antibodies (green histograms) is indicated in the M1 region of each plot.

7R$^+$), IL-7R was not expressed on a significant proportion of HIV-specific CD8 cells, suggesting that lack of IL7-R expression was specific to the HIV-specific cells and was not a universal feature of all CD8 cells. CD25 expression was absent from both the total CD8 population and the HIV-specific CD8 cell population.

Figure 5.14 shows the results obtained when QIYAGIKVK-specific cells cryopreserved from patient MM12 at series of timepoints were stained to reveal the expression of IL-7R and CD25 on tetramer positive cells. It can be seen that staining for these two markers was absent at all timepoints. An absence of CD25 and IL-7R expression on HIV-specific T cells was a common feature of all responses studied (Table 5.2), except for the KAFSPEVIPMF-specific response in patient MM13 at very late timepoints where there was a low proportion of cells that expressed CD25.

5.5.3 Expression of markers indicative of the proliferative status and terminal differentiation of HIV-specific CD8$^+$ T cells

A desirable feature of a virus-specific CD8$^+$ T cell response, particularly in the context of chronic infection, may be the presence of cells which possess a strong proliferative capacity. In order to look features of HIV-specific CD8$^+$ T cells associated with their division and replicative history, the levels of Ki67 and CD57 expression on the cells were assessed. Ki67 is an intracellular marker of proliferation and so the proportion of Ki67$^+$ cells can be used as a measure of T cell division. On the other hand, CD57 is reported to be a marker on cells that have undergone multiple rounds of cell division and are unable to proliferate after antigen-specific stimulation *in vitro*, and undergo activation-induced apoptosis (Brenchly *et al.*, 2003).

When the expression of Ki67 and CD57 on HIV-specific cells and the total CD8 population in patient MM12 at 40 DFOSx were compared (Figure 5.15), it was apparent that a higher percentage of HIV-specific cells expressed the two markers than did CD8 cells in the total CD8 pool, which is consistent with the HIV-specific cells having undergone more rounds of division and being less capable of further proliferation than most CD8$^+$ T cells of other specificities, a probable consequence of chronic antigenic stimulation.

Changes in expression of Ki67 and CD57 on A3 QIYAGIKVK-specific cells as the response evolved in MM12 are shown in Figure 5.16. During the expansion phase of the response, the majority of tetramer positive cells were CD57$^-$ and a considerable proportion (~30% of tetramer positive cells) showed

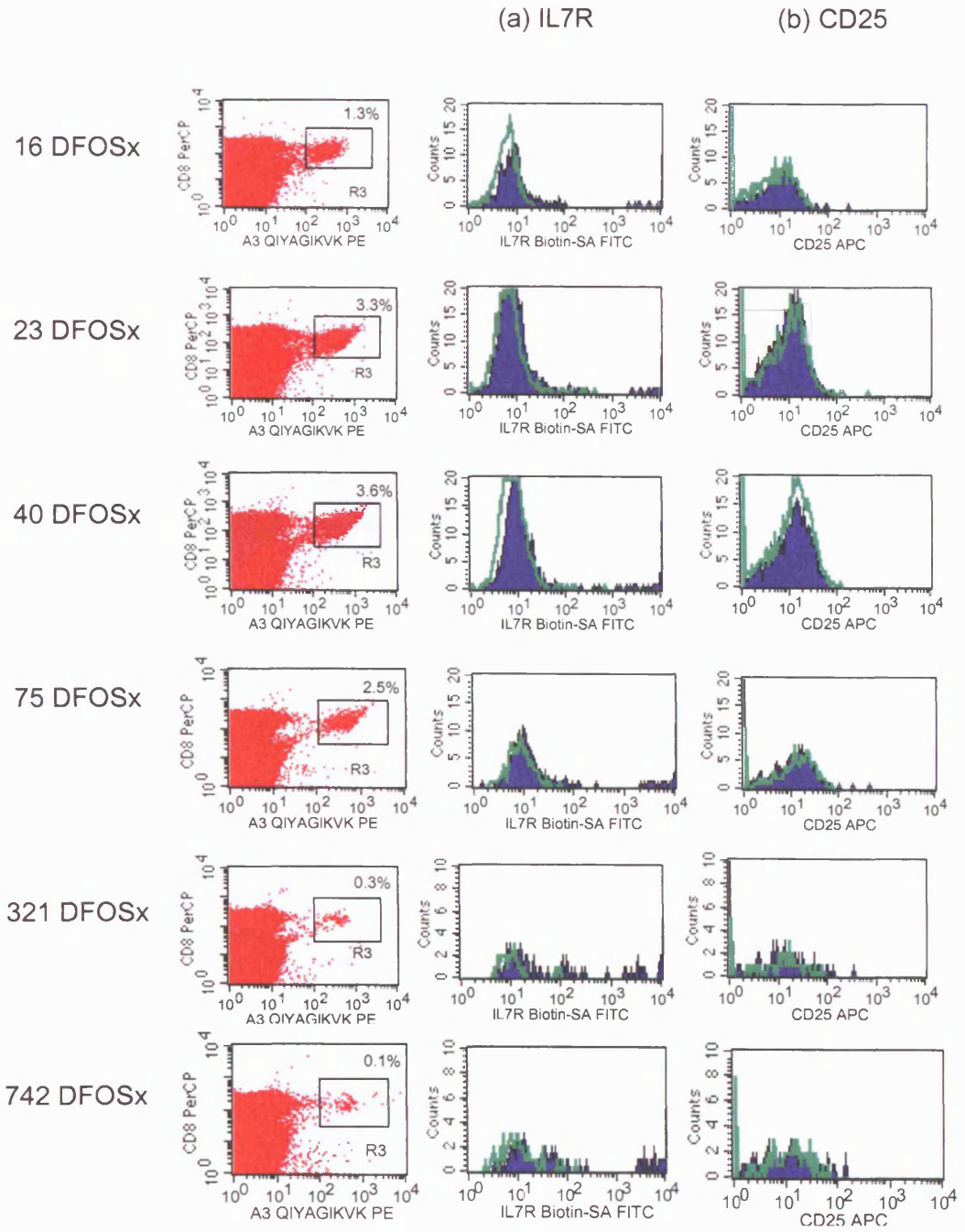


Figure 5.14. Expression of CD25 and IL-7R on A3 QIYAGIKVK-specific CD8$^+$ T cells during the course of HIV infection. Cells cryopreserved from patient MM12 at 16, 23, 40, 75, 321 and 742 DFOSx were co-stained with an A3 QIYAGIKVK-PE tetramer and an IL7R biotin-SA FITC or CD25 APC antibody. Each dot plot is gated on the CD8$^+$ tetramer$^+$ population shown on the corresponding dot plot shown on the left. The histogram plot at each timepoint shows the levels of expression of the marker on the tetramer positive population (blue histogram) above background staining of the cells with an appropriate isotype control antibody (green line). The percentages of CD8$^+$ tetramer$^+$ cells (in the R3 region) at each timepoint are given.

Patient	Specificity of $CD8^+$ T cells	Timepoint at which cells were taken for phenotyping (DFOSx)	Phenotype of HIV epitope-specific cells at indicated timepoint	
			IL-7R	CD25
MM25	A3 RLRPGGKKK	17	-	-
		31	-	-
		66	-	-
		185	-	-
MM12	A3 QIYAGIKVK	16	-	-
		23	-	-
		40	-	-
		75	-	-
		321	-	-
		742	-	-
	B7 IPRRIRQGL	139	-	-
		230	-	-
		567	-	-
MM13	B8 FLKEKGGL	16	-	-
		31	-	-
		196	-	-
		544	-	-
	B57 KAFSPEVIPMF	98	-	-
		196	-	±
		544	-	±
SUMA	B15 MTKGLGISY	8/15	-	-
		29/41	-	-
	B14 ERYLKDQQL	22	-	-
		483	-	-

Symbols represent percentage of $CD8^+$ $tetramer^+$ cells expressing a given marker:
- 0% - 9%
± 10% - 19%
+ 20% - 39%
++ 40% - 59%
+++ 60% - 79%
++++ 80% - 89%
+++++ 90% - 100%

Table 5.2. Expression of cytokine receptors on HIV-specific $CD8^+$ T cells over the course of infection. PBMC cryopreserved from patients MM25, MM12, MM13 and SUMA at different stages of infection were stained with the indicated tetramers (or pentamers) to identify HIV-specific $CD8^+$ T cells responding to one or two different viral epitopes, and were co-stained with fluorescently labelled antibodies specific for CD8 and IL-7R or CD25 to allow assessment of the phenotype of epitope-specific T cells. The percentage of $CD8^+$ $tetramer^+$ cells expressing each marker was assessed by flow cytometry, and is represented in the table by + and – signs as explained in the key.

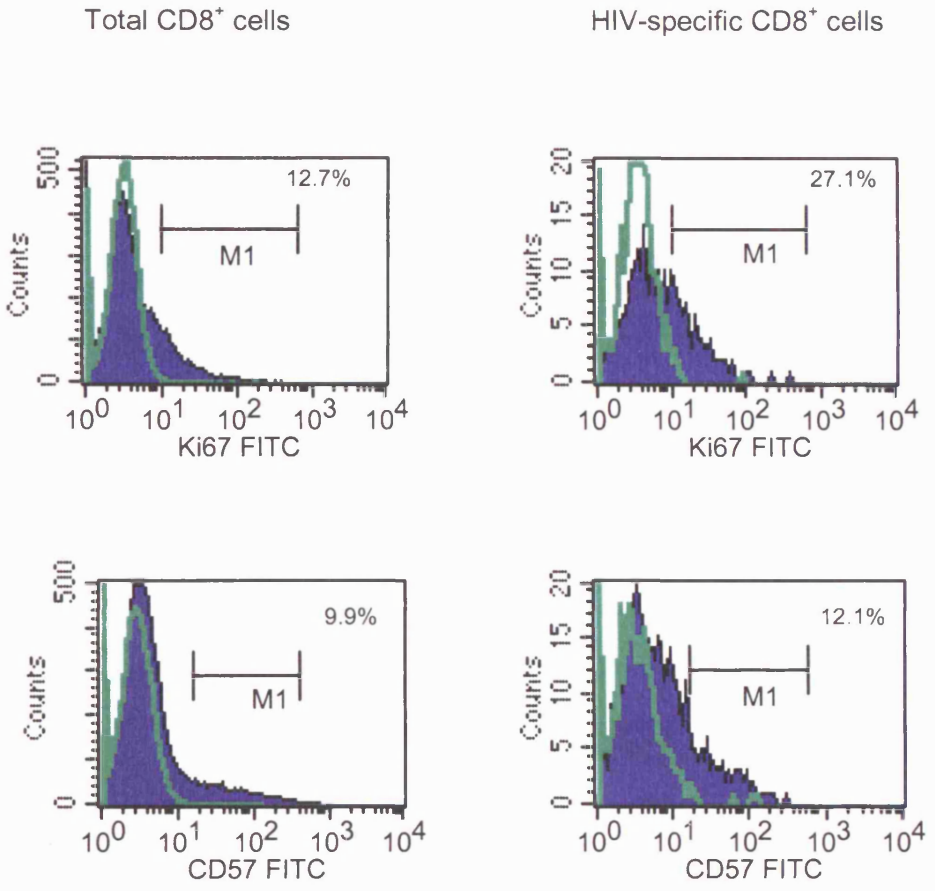


Figure 5.15. Ki67 and CD57 expression on total CD8$^+$ cells and HIV-specific CD8$^+$ cells from patient MM12. Cells cryopreserved from patient MM12 at 40 DFOSx were co-stained with an A3 QIYAGIKVK tetramer and antibodies specific for CD8 and either Ki67 or CD57. Plots on the left are gated on the CD8$^+$ lymphocyte population, and those on the right on the CD8$^+$ tetramer$^+$ population. The proportion of cells in the total CD8$^+$ lymphocyte population or the CD8$^+$ tetramer$^+$ population expressing each of the markers (blue histograms) at levels above the background staining of cells incubated with appropriate isotype control antibodies (green histograms) is indicated in the M1 region of each plot.

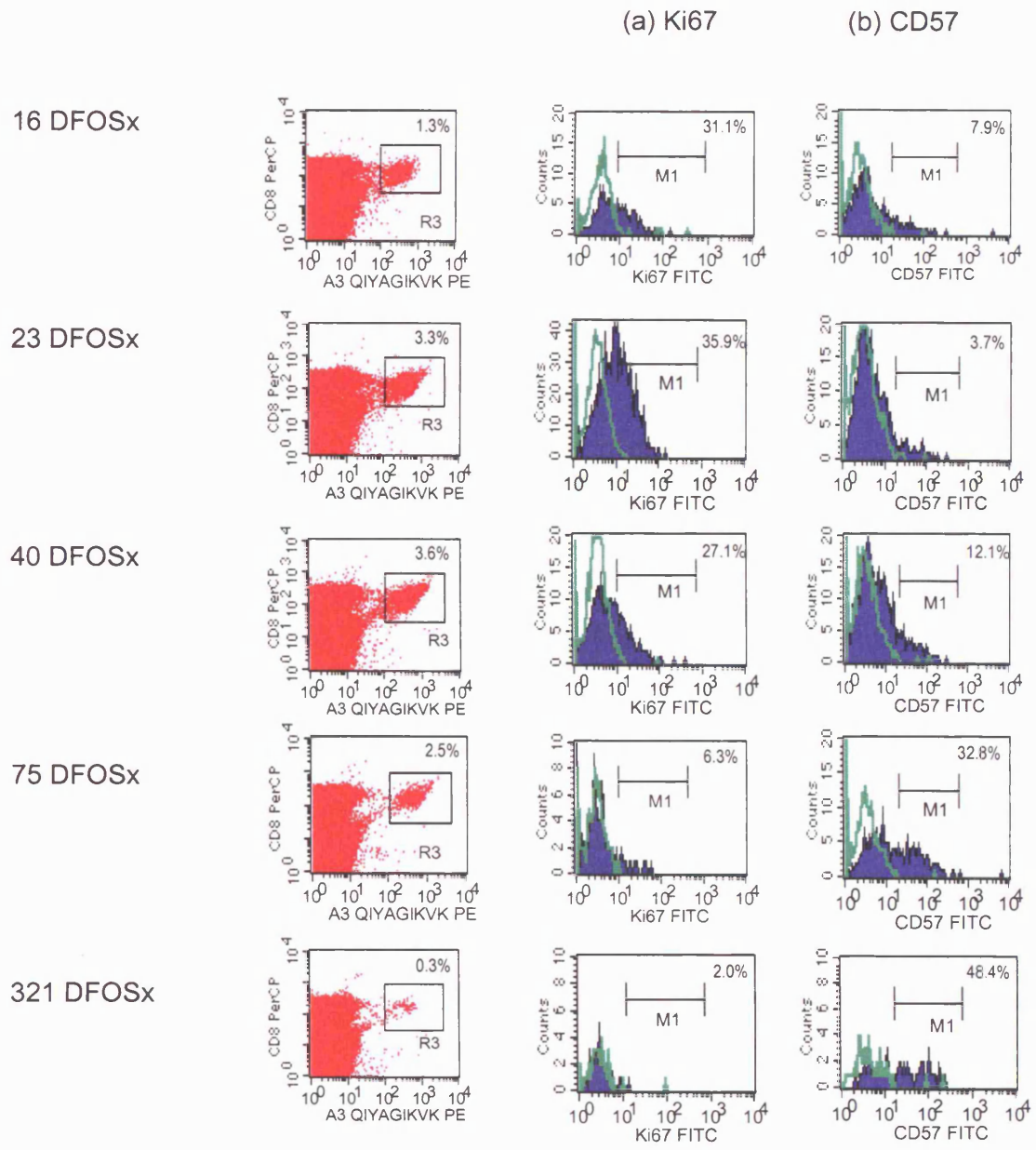


Figure 5.16. Expression of Ki67 and CD57 on A3 QIYAGIKVK-specific CD8$^+$ cells during the course of HIV infection. Cells cryopreserved from patient MM12 at 16, 23, 40, 75 and 321 DFOSx were co-stained with an A3 QIYAGIKVK-PE tetramer and anti-CD8 antibody and (a) Ki67-FITC antibody or (b) CD57-FITC antibody. Each histogram plot is gated on the CD8$^+$ tetramer$^+$ population shown in the R3 region in the corresponding dot plot shown on the left. The histogram plot at each timepoint shows the levels of expression of the marker on the tetramer positive population (blue histogram) above background staining of the cells with an appropriate isotype control antibody (green line). The percentages of CD8$^+$ tetramer$^+$ cells (in the R3 region) at each timepoint are given, as are the percentages of CD8$^+$ tetramer$^+$ cells expressing Ki67 or CD57 (in the M1 region).

Ki67 expression. Later on, after the response had peaked, the cells lost Ki67 expression, and over time, an increased frequency of epitope-specific cells were found to express CD57 (48% of tetramer positive cells were $CD57^+$ by 321 DFOSx). This pattern of expression of the two markers is typical of what was also seen for the majority of the responses studied (Table 5.3). Expression of Ki67 during the expansion phase of the response is likely a reflection of intense proliferation of antigen-specific cells during acute infection; the proportion of $Ki67^+$ cells subsequently decreases as the response stabilises and/or contracts. Increases in the proportion of cells expressing CD57 with time may reflect chronic antigenic stimulation of cells promoting continuous rounds of cell division and eventual terminal differentiation of antigen-specific cells. IPRRIRQGL-specific cells were $CD57^+$ at all timepoints studied, as the response was only examined at timepoints when the response was in decline. The interesting exception to this was in patient MM13 in whom very few KAFSPEVIPMF-specific cells exhibited Ki67 expression and a moderate level displayed CD57, yet cells were phenotyped at timepoints when this response was in the earlier stages of expansion. This may be explained by the observation that this response showed an unusually slow expansion, over a very long period of time (it was still increasing at the latest timepoint studied, which was two years into infection), so the proportion of cells that were proliferating was low (hence the very low levels of Ki67 expression), and some cells had probably already undergone many rounds of division over this time, and their replicative lifespan was coming to an end (hence the moderate levels of $CD57^+$ cells).

5.6 Discussion

During primary HIV infection, there is an acute burst of viral replication, associated with which, a virus-specific $CD8^+$ T cell response is induced (Borrow *et al.*, 1994; Koup *et al.*, 1994). However relatively few studies have examined the virus-specific T cell response in primary HIV infection, and many questions remain unanswered. Notably, our understanding of the magnitude and kinetics of the initial expansion of response(s) in acute infection is very limited; and whether or not multiple epitope-specific responses all expand together in primary infection, or whether different epitope-specific responses expand asynchronously (and if so, whether the later-expanding responses are subdominant responses), is also unclear. Also, how do these parameters differ

Patient	Specificity of $CD8^+$ T cells	Timepoint at which cells were taken for phenotyping (DFOSx)	Phenotype of HIV epitope-specific cells at indicated timepoint	
			Ki67	CD57
MM25	A3 RLRPGGKKK	17	++	-
		31	±	-
		66	±	±
		185	-	+
MM12	A3 QIYAGIKVK	16	+	-
		23	+	-
		40	+	±
		75	-	+
		321	-	++
		742	N.D.	N.D.
	B7 IPRRIRQGL	139	N.D.	+
		230	N.D.	+
		567	N.D.	+
MM13	B8 FLKEKGGL	16	++	-
		31	+	++
		196	-	+
		544	N.D.	+
	B57 KAFSPEVIPMF	98	-	+
		196	-	+
		544	-	+
SUMA	B15 MTKGLGISY	8/15	+	-
		29/41	-	±
	B14 ERYLKDQQL	22	+	-
		483	-	+

Symbols represent percentage of $CD8^+$ $tetramer^+$ cells expressing a given marker:

- 0% - 9% ± 10% - 19% + 20% - 39%
++ 40% - 59% +++ 60% - 79%
++++ 80% - 89% +++++ 90% - 100%

N.D. not determined

Table 5.3. Changes in expression of markers of cell turnover on HIV-specific $CD8^+$ T cells over the course of infection. PBMC cryopreserved from patients MM25, MM12, MM13 and SUMA at different stages of infection were stained with the indicated tetramers (or pentamers) to identify HIV-specific $CD8^+$ T cells responding to one or two different viral epitopes, and were co-stained with fluorescently labelled antibodies specific for CD8 and Ki67 or CD57 to allow assessment of the phenotype of epitope-specific T cells. The percentage of $CD8^+$ $tetramer^+$ cells expressing each marker was assessed by flow cytometry, and is represented in the table by + and – signs as explained in the key.

between patients, and importantly, if there are differences, how do these relate to the persisting viral load established?

To address these questions, tetramers were used to stain PBMC samples cryopreserved at a series of timepoints throughout the course of acute and/or early infection from different infected individuals to reveal the magnitude and kinetics of expansion of different epitope-specific responses. A number of different tetramers were used, to allow comparison of several responses within the same patient, and comparison of responses in patients who went on to establish different persisting viral loads. The use of tetramer staining allows accurate assessment of the total magnitude of individual responses, as tetramers detect epitope-specific cells regardless of their functional capacity. This may be especially important in primary infection where many cells are highly activated and likely to die on *in vitro* restimulation. Such cells may not be detected by other methods which rely on cells to be able to perform a specific function, such as in IFN-γ based ELISPOT assays.

One of the issues I addressed was the magnitude of responses to individual viral epitopes during primary HIV infection. When tetramer staining was used to follow a number of HIV epitope-specific responses in seven patients the highest recorded magnitudes of the responses studied ranged from 22% to 0.2% of $CD8^+$ cells. Whether the highest recorded magnitude of a response represents the actual peak of the response is uncertain, since tetramer staining could only be carried out at timepoints from which PBMC samples were available (typically spaced ~one week apart), so it is possible that responses may have actually peaked at a timepoint when a sample was not available for testing. Nonetheless, these results indicate that responses to immunodominant HIV epitopes can reach very high magnitudes during primary infection.

In line with this, when Pantaleo *et al* used a semi-quantitative PCR approach to look at changes in patterns of TCR Vβ family usage by PBMCs of HIV-infected individuals during acute/early infection, in one patient a major expansion in $V\beta19^+$ cells was observed, comprising 40% of PBMC (Pantaleo *et al.*, 1994). It was likely that the 40% of PBMCs expressing this single Vβ family (Vβ19) corresponded to cells responding to a single highly immunodominant epitope found to be recognised in the same patient by Borrow *et al* (Borrow *et al.*, 1997). The total frequency of epitope-specific cells may thus have been greater than 40% of all CD8 cells in this patient at/near

the peak of the response. The limited studies which have previously been done using tetramers to enumerate the primary HIV-specific CD8 response have indicated that epitope-specific T cell frequencies reach up to between 2% and 5% of CD8 cells (Appay, 2002; Wilson *et al.*, 2000), which is within the range observed in the patients I studied. As the patients progress to the chronic stage of infection, responses are typically reported to be of lower magnitude, but responses against individual epitopes which comprise 1-2% of CD8 cells are still common (Altman *et al.*, 1996; Ogg *et al.*, 1998).

The total magnitude of the primary HIV-specific CD8 T cell response was more difficult to estimate from the analysis I carried out, since responses to only a few epitopes were studied using tetramers in each patient. Although results in chapter 3 indicate what the most dominant responses were in early infection in each patient, it is unclear which of these was the most dominant during acute/subacute infection; plus it is also possible that there may have been responses to other epitopes that were not detected due to differences between the patient's autologous virus sequence and the sequence of the consensus peptides used for epitope identification. However minimum estimates of the total magnitude of the HIV-specific CD8 response can still be made from results here. In patient SUMA, at least 25% of CD8 cells were likely HIV-specific at/near the peak of the response (considering the combined magnitude of the responses to the B15 MTYKGLGISY and B14 ERYLKDQQL epitopes). In patient MM25 the total HIV-specific response reached a maximum magnitude of at least 14% of all CD8 cells (again, this was calculated by combining the percentages of cells specific for two epitopes). However, in the latter patient it is notable that the two epitopes which were studied using tetramers corresponded to only two of four dominant responses identified in this patient (Figure 3.11c). Although a possible optimal epitope was identified within a third of the four long peptides to which a strong response was seen in early infection in this patient, a tetramer corresponding to this epitope could not be obtained. The optimal epitope was not able to be predicted for the fourth dominant response. In addition, it was possible that another epitope was contained within the long peptide which corresponded to the RPQVPLRPMTY epitope. Consequently, it is likely that the overall primary HIV-specific response in MM25 was much larger than the minimal estimate obtained by using two tetramers to study the response.

One of the questions it was hoped these results may answer was whether the total magnitude of the primary HIV-specific $CD8^+$ T cell response differs in

patients who subsequently establish different persisting viral loads. Due to reasons already mentioned, this cannot be answered with confidence; but from the minimum total responses that were detected, it appears that a high magnitude response alone doesn't predict good clinical outcome. Both patients SUMA and MM25 made large magnitude responses, but they controlled early viral replication with quite different efficiencies. Further evidence suggesting that high magnitude primary HIV-specific T cell responses may be mounted in patients who establish high persisting viral loads is provided by the studies of Pantaleo *et al* mentioned above (Pantaleo *et al.*, 1994; Pantaleo *et al.*, 1997b), where large distortions of the T cell repertoire indicative of expansion of HIV-specific T cell responses were observed during primary infection in multiple patients who subsequently contained viral replication poorly.

High magnitude primary virus-specific $CD8^+$ T cell responses have been described in other viral infections too, such as in LCMV infection in mice where 50% of $CD8^+$ cells in $H\text{-}2^d$ mice can be directed against one dominant epitope at the peak of the acute response, and the total LCMV-specific response can comprise almost 63% of all CD8 cells (Murali-Krishna *et al.*, 1998); likewise in primary EBV infection, a massive expansion of cells specific for a single epitope has been observed, comprising 44% of $CD8^+$ T cells (Callan *et al.*, 1998).

By contrast, in the acute phase of some other viral infections, while significant populations of virus-specific $CD8^+$ cells have been observed at/near the peak of the primary response, these have not been found to be as large as those described above for LCMV, HIV and EBV. Studies of epitope-specific responses in primary hepatitis C infection have commonly reported frequencies of tetramer-positive cells of up to ~ 3% of CD8 cells (Gruener *et al.*, 2001; Kantzanou *et al.*, 2003; Lechner *et al.*, 2000b; Urbani *et al.*, 2002), although two studies were able to detect HCV-specific responses of around 7% (Lechner *et al.*, 2000a) and 20% of CD8 cells (Timm *et al.*, 2004).

The magnitude of the virus-specific response detected during primary HBV infection has only been reported to reach levels of ~1% of $CD8^+$ T cells (Maini *et al.*, 1999; Urbani *et al.*, 2002).

These differences in the magnitude of the virus-specific CD8 responses in different viral infections may potentially reflect differences in the tissue sites of replication of the virus, which may affect the size of response induced and/or the distribution of virus-specific T cells. For example LCMV and HIV are both

viruses which replicate systemically in lymphoid tissues so it is perhaps not surprising that they are able to elicit such high magnitude $CD8^+$ T cell responses. Replication within the immune system, in B cells, may also contribute to the antigenicity of EBV. By contrast HBV and HCV infections preferentially replicate in the liver. However a high magnitude virus-specific $CD8^+$ T cell response is induced in influenza virus infected mice, despite the fact that the replication of this virus is confined to the respiratory tract (Belz *et al.*, 2001; Marshall *et al.*, 2001). In addition, virus-specific T cell frequencies are commonly measured in peripheral blood (or lymphoid tissues in mice). Virus-specific $CD8^+$ T cells sequestered at tissue sites of viral replication may thus go undetected, resulting in an underestimation of the magnitude of the total virus-specific response, particularly in those infections where virus replication is confined to, for example, the liver.

Another factor which may influence the activation of a virus-specific $CD8^+$ T cell response is the kinetics of viral replication. Hepatitis viruses replicate very slowly by comparison to viruses such as LCMV. Measurements of LCMV epitope-specific T cell responses induced by different strains of LCMV showed that slowly replicating strains induced weaker responses than more rapidly replicating strains (Bocharov *et al.*, 2004). It was thus proposed that the speed of viral replication may influence the initial strength of the CD8 response. This may explain the weaker responses observed in infection with slowly replicating viruses such as HBV and HCV.

Another issue I addressed was the kinetics of expansion of epitope-specific $CD8^+$ T cell responses in primary infection. The kinetics of expansion of one to four different epitope-specific responses were followed in relation to the kinetics of viral replication in patients who established different persisting viral loads. There were some limitations to this analysis - without daily samples, the precise kinetics of viral replication and expansion of CD8 responses could not be determined (i.e. the timepoints at which viral replication and the frequency of epitope-specific cells peaked could only be estimated from available data, as values at intermediate timepoints were unknown). Also, due to not having tetramers corresponding to all viral epitopes recognised during the primary HIV-specific $CD8^+$ T cell response in each individual, it was not certain that the first epitope-specific response to expand in a given patient was studied here. Nonetheless, some interesting observations emerged from this analysis.

The time between the estimated peak viral load and the highest recorded CD8 expansion was determined for each epitope-specific response followed. The day at which the peak in viraemia occurred was taken to be either that when the highest viral load was recorded (at 7 DFOSx for MM25, 6 DFOSx for MM28 and 7 DFOSx for MM12 and 8 DFOSx for SUMA) or if it was clear that the highest viral load recorded was not the peak viral load, 9 DFOSx was taken to be a reasonable estimate (for MM27, MM14), except in the case of MM13 in whom it was apparent that the peak in acute viral replication must have occurred prior to 9 DFOSx. It may have been possible that the virus replicated with slightly faster kinetics in MM13 compared to the average patient; alternatively, the recorded day of onset of symptoms may not have been accurate. In this patient, 7 DFOSx was taken as an estimate of the day at which the viral load may have peaked.

The shortest time between the peak viral load and peak CD8 expansion was that observed in patient SUMA where the highest value for the MTKGLGISY-specific response was recorded at the same timepoint as for when the highest viral load was recorded (8 DFOSx). Due to the timepoints at which epitope-specific T cell frequencies were evaluated, it was possible that the response may have peaked anywhere between -4 days and +7 days relative to the highest recorded viral load – but even using the latter estimate, this was a rapidly-expanded response. The slowest response to evolve was seen in MM19 in whom the response to KAFSPEVIPMF expanded with very slow kinetics and was still rising at the last timepoint studied (189 days after the estimated time at which the peak viral load occurred). Taking into consideration all epitope-specific responses studied, the average time between the peak viral load and maximum epitope-specific response was 62 days. Perhaps more importantly, the average time between the peak viral load and the point when the most rapidly-expanding of the responses studied in a given individual reached its maximum recorded value was 39 days.

Compared with some other viral infections, the HIV-specific response thus appears to expand with relatively delayed kinetics. In the LCMV model, the precise kinetics of viral replication and expansion of CD8 responses have been studied, and LCMV-specific responses have found to be mounted very rapidly following infection, and typically peak within a few days following the peak in acute viral replication (Lau *et al.*, 1994). Similarly, during primary SIV_{mac} infection of rhesus monkeys, a rapid expansion of an epitope-specific

response was observed to occur, which peaked approximately four days after the peak of viraemia (Kuroda *et al.*, 1999).

Data about the kinetics of virus-specific $CD8^+$ T cell expansion relative to primary viral replication in human viral infections other than HIV-1 is relatively incomplete. This is due in part to insufficient monitoring of responses over the acute/early stages of infections; the lack of acute/early viral load data; and the fact that often T cell responses of only chosen specificities are studied, and it is possible that subjects develop more significant early responses against antigenic specificities not tested.

However there are some studies which suggest that HBV and HCV infection may be similar to HIV in that virus-specific $CD8^+$ T cell responses are relatively slow to develop after infection. The few studies where longitudinal analyses of viral load and epitope-specific responses were carried out show that there may be a delay of possibly several weeks between the peak in viraemia and peak CD8 response in HBV and HCV infections (Thimme *et al.*, 2001; Webster & Bertoletti, 2002).

HIV, HBV and HCV are all viruses which persist in their hosts, so it possible that the delay in expansion of the virus-specific $CD8^+$ T cell response may one factor which may contribute to the failure of the host to successfully clear these infections. Studies in murine models have shown that small changes in the speed and efficiency of CTL inhibition of viral replication can have profound effects on viral control (Ehl *et al.*, 1997). Consistent with this idea is the finding that there is a delay in activation of antigen-specific $CD8^+$ T cells following SHIV challenge of vaccinated monkeys, which is thought to allow for unimpeded early viral replication and contribute to the inability of vaccine-elicited T cell responses to prevent establishment of persistent infection (Davenport *et al.*, 2004).

Notably, although the number of patients I studied was small, and the possibility that potentially significant epitope-specific responses were not followed in some subjects means that definitive statements cannot be made as to which patients had the fastest/slowest evolving responses, it is interesting that the response which expanded with the most rapid kinetics was observed in the patient who controlled early viral replication the most efficiently (the MTKGLGISY-specific response in SUMA). The rapid mobilisation of a high magnitude virus-specific $CD8^+$ T cell response in patient SUMA may have been one of the factors contributing to the ability of this

patient to control early viral replication very efficiently (although cause and effect are hard to distinguish).

To explore this idea further, more comprehensive studies of the kinetics of expansion of epitope-specific $CD8^+$ T cell responses in larger numbers of infected individuals would need to be carried out. It would also be interesting to compare the kinetics of the virus-specific response in the minority of HCV-infected patients who are able to spontaneously clear acute infection to that in patients who are unable to clear the virus and establish a chronic infection. Is the more favourable outcome in those who clear the virus related to the ability to mount a faster HCV-specific response? One study did look at the primary virus-specific $CD8^+$ T cell response in patients representing the two different outcomes of infection (Thimme *et al.*, 2001), but the results did not enable a fair comparison of the kinetics of expansion of $CD8^+$ T cell responses, for the chronically infected patient failed to induce a significant T cell response that was amenable to detection using the tetramers used for following HCV-specific responses (although if this patient in fact failed to induce a significant response at all, this could itself have been responsible for viral persistence).

The availability of tetramers to follow more than one epitope-specific response within a particular patient allowed for the study of the synchronicity with which different epitope-specific responses expanded in six patients. In four of these individuals (SUMA, MM28, MM13 and MM12), the responses expanded asynchronously, with some responses expanding at earlier timepoints, and others later. In the other two patients (MM27 and MM25), the epitope-specific responses studied both expanded and contracted simultaneously; however there may have been other responses not studied in these individuals which exhibited a different pattern of expansion. It may thus potentially be the case that responses expand asynchronously in all patients.

The asynchronous expansion of epitope-specific T cell responses observed during the first ~six months of HIV-1 infection likely reflects a combination of differing rates of initial activation/proliferation of epitope-specific T cells and subsequent shifts in the hierarchy of responses to different viral epitopes as a result of antigenic changes in the persisting viral quasispecies.

The asynchronous initial expansion of different epitope-specific responses in HIV infection is in contrast to what is seen in LCMV infection. Here the initial pattern of expansion of responses is one in which dominant and subdominant

responses all expand and decline in parallel (Murali-Krishna *et al.*, 1998) (although if the virus persists, the immunodominance hierarchy can change over time (Wherry *et al.*, 2003a)). Further, in a (less comprehensive) study of the expansion of T cell responses of more than one specificity in two patients with self-limited primary HBV infection, a synchronous pattern in the evolution of responses was observed during primary infection (Maini *et al.*, 1999). By contrast the data available from HCV infection suggest that epitope-specific responses do not expand in parallel in this infection (Lechner *et al.*, 2000a).
Factors that may underlie the preferential expansion of certain T cell populations in primary HIV infection were discussed in chapter 4. It was hypothesised there that limited availability of activated APCs and/or $CD4^+$ T cell help (which may also be features of primary HCV infection) may result in fierce competition between HIV-specific T cells, and initial expansion of T cells with the least stringent activation requirements (e.g. cells responding to high affinity epitopes and/or previously primed T cells). It was notable that the responses which evolved with the most rapid kinetics in primary HIV infection were typically dominant responses. In agreement with this are observations made in other studies, which likewise found that subdominant responses had a slower rate of expansion (Davenport *et al.*, 2004; De Boer *et al.*, 2001). Studies in which control and IFN-γ deficient mice were immunised with DNA vaccines encoding dominant and subdominant LCMV epitopes demonstrated that subdominant T cell responses can be suppressed by dominant CD8 T cell responses in an IFN-γ-dependent manner (Rodriguez *et al.*, 2002). This mechanism could also contribute to the immunodominance of those HIV-specific T cell populations that initially start to expand first in primary HIV infection.

Delayed expansion of responses of certain epitope specificities may reflect changes in the epitopes presented to virus-specific T cells at different stages of infection. For example in EBV infection, the kinetics of expansion of responses to lytic and latent antigens are different, with responses to lytic epitopes dominating during the primary EBV-specific response (when a lot of productive viral replication is occurring) but these responses subsequently declining, and responses to latent epitopes being proportionately increased after the transition to persistent infection occurs (Hislop *et al.*, 2002). In HIV infection, marked changes in the pattern of viral protein production do not occur at different stages of infection; however the composition of the viral

quasispecies does change over time. Amino acid changes can be selected for in/around $CD8^+$ T cell epitopes that confer escape from the epitope-specific T cell response – presentation of certain viral epitopes may thus be markedly diminished within as little as one-two months following the acute viral burst (Borrow *et al.*, 1997; Jones *et al.*, 2004). The magnitude of the $CD8^+$ T cell response to these epitopes may then decline, enabling expansion of responses to previously subdominant epitopes – i.e. viral escape may drive shifts in the specificity of the HIV-specific $CD8^+$ T cell response (Nowak *et al.*, 1995). This may explain the differences in the specificity of the HIV-specific $CD8^+$ T cell response observed in the primary and chronic phases of infection (Altfeld *et al.*, 2001a; Dalod *et al.*, 1999b; Goulder *et al.*, 2001a), for example the delayed appearance of the A2-restricted response directed towards the SLYNTVATL epitope despite the presence of this epitope in the infecting viral strain (Goulder *et al.*, 2001a).

The clonal composition of different HIV-specific $CD8^+$ T cell responses as they evolved was also studied in this chapter. Results from analysis of the clonal composition of HIV epitope-specific $CD8^+$ T cell responses at one timepoint (at or near the peak of their expansion) in chapter 3 suggested that responses tended to involve cells expressing a limited range of Vβ families. This led to the question of whether the response was initially broad and certain TCRs were selected for as the response expanded to its peak magnitude, or whether a limited response was initially raised? A further question of interest was whether the same Vβ families were used in a given response over time, or whether the response evolved to involve new populations of T cells? TCR Vβ family usage by epitope-specific cells was assessed at different timepoints during the course of infection in different patients to address such questions. Whether there were any differences in the pattern of Vβ family usage in responses that expanded early and later, and between responses in patients who controlled viral replication with differing efficiency was also addressed. As discussed in chapter 3, limitations of using this approach to assess the TCR usage by epitope-specific T cells include the fact that it does not provide information about the range/identity of T cell clones within a given Vβ family involved in the response; also there were certain Vβ families for which no antibodies were available, which meant that if a large proportion of the response remained unaccounted for using the antibodies available, it could

not be determined whether the remainder of the response was broad or highly focused in terms of Vβ family usage.

Results from studying responses to seven epitopes in four patients suggested that great heterogeneity of TCR use in HIV-specific responses was uncommon: for only one response was there a heterogeneous TCR usage observed which remained heterogeneous over time (QIYAGIKVK-specific T cells in MM12 (Figure 5.6)). There were three examples of responses which were of moderate heterogeneity at the first timepoint studied (although with a more prominent component to the response in each case), and which then exhibited greater focusing towards one particular Vβ family over time (KAFSPEVIPMF-specific T cells in MM13 and MTYKGLGISY-specific and ERYLKDQQL-specific response in patient SUMA, although a large proportion of the two latter responses were undetected using the available Vβ family antibodies). There were two examples of responses (Figures 5.5 and 5.6) which were more narrowly focused from the start and in which the dominant TCR Vβ family used changed over time, the RLRPGGKKK-specific response in MM25 showing the more rapid and dramatic change. Another response (FLKEKGGL-specific response in MM13 (Figure 5.7)), was also focused on a limited number of Vβ families, but a stable response to the dominant Vβ family used was seen at all timepoints.

This leads to the question of why, in five out of seven epitope-specific responses studied, does TCR usage change over time? An important consideration in this respect is that the composition of the viral quasispecies may be changing, and so the observed change in TCR usage could be a reflection of alterations in the stimulation of different populations of epitope-specific T cells by variant epitope sequences. However, the changing TCR usage may be a consequence of clonal exhaustion or selection for certain clones of cells. Clones of T cells which disappear by exhaustion would be replaced by other clones of cells within the epitope-specific population, and this may lead to changes in the TCR usage of epitope-specific cells observed (Pantaleo *et al.*, 1997a). If selection for T cell clones utilising a specific Vβ family is occurring, this leads to the question of why, and whether there are immunological benefits to be gained?

Greater heterogeneity of TCR usage in an epitope-specific CD8 response may be beneficial by allowing recognition of variant versions of the epitope peptide, and reducing the capacity for immunological escape by the virus via the acquisition of mutations that may reduce T cell recognition (Lopes *et al.*,

2003). However, biasing of TCR usage towards a single receptor, the use of which is associated with a beneficial outcome, might also be advantageous. For example, the selected TCR may have a high capacity to recognise potential epitope variants (Dong *et al.*, 2004), or perhaps have a higher affinity for its ligand and contribute to a higher avidity response (Busch & Pamer, 1999; Savage *et al.*, 1999).

To investigate whether T cells expressing high avidity TCRs were selected for during the course of infection (resulting in the changes in Vβ usage by epitope-specific T cells observed to take place in some cases), the functional avidity of epitope-specific T cells at an earlier and a later timepoint during evolution of a response were determined. One limitation to this approach is that it was assumed that the peptide(s) used in experiments to determine functional avidity corresponded to the autologous viral peptide sequence, but this was not known for certain. Therefore alterations in the avidity of the response as measured *in vitro* using non-autologous viral sequence may not necessarily be representative of the avidity of the response *in vivo*.

For the FLKEKGGL-specific response in MM13, where Vβ usage was relatively stable, there was no change in the avidity of the T cell response with time. However, for the RLRPGGKKK-specific response in MM25, for which there was gradually more selection for Vβ2 usage with time, there was an increase in avidity of the response by an order of two logs, such that the cells at the later timepoint were now sensitive to a much lower level of peptide. That the higher avidity cells had a greater percentage of Vβ2$^+$ T cells suggests that for this response, there was correlation of Vβ2 usage with higher avidity, and that TCRs utilising this particular Vβ family may have been associated with improved interaction with the peptide-MHC complex, contributing to a higher avidity response. This could have been confirmed by making T cell lines/clones of Vβ2$^+$ and Vβ2$^-$ epitope-specific T cells and comparing their avidities. However, there are also other factors which could influence the avidity of a T cell response. For example, in addition to changes in TCR usage, an increase in the expression of adhesion or co-stimulatory molecules on T cells may contribute to an increase in the avidity of the response.

A possible advantage to the host of using cells with a higher functional avidity is that higher avidity CTL may be more effective than lower avidity CTL at combating viral infection, as several studies have shown (Alexander-Miller *et al.*, 1996; Gallimore *et al.*, 1998; Sedlik *et al.*, 2000). Higher avidity CTL were shown to be more efficacious at preventing spread of virus as they were able

to recognise infected cells earlier, at a time when little viral protein had been made and few new virions produced (Derby *et al.*, 2001).

Why should a low avidity response be induced to some viral epitopes initially, if the host has the capacity to mount a higher avidity response? Changes in the avidity of a HIV-specific response may be a reflection of improved APC function later during the course of infection, enabling higher avidity T cells to expand. Studies have shown that CD4 help and high levels of co-stimulation have a role in the induction of high avidity CTL (Oh *et al.*, 2003). Clinical data for patient MM25 show that there was not a substantial difference in the CD4 count at the two timepoints when the avidity of the RLRPGGKKK-specific response was measured, but it is feasible that improved APC function and/or late induction or recovery of function of HIV-specific $CD4^+$ T cells might have contributed to the increased avidity of the $CD8^+$ T cell response to this epitope.

A further series of experiments explored the phenotypic characteristics of tetramer binding cells, using four-color flow cytometry. The majority of HIV-infected individuals are unable to control viral replication despite the presence of large numbers of HIV-specific $CD8^+$ T cells. It is suggested that this may be due in part to failure of these cells to mature into fully differentiated effector cells.

The differentiation of $CD8^+$ T cells has been the subject of many studies, but is still not fully understood, and it is debated as to what the lineage relationship between phenotypically distinct subsets of $CD8^+$ T cells may be.

Naïve cells are defined by the phenotype $CD45RA^+$ $CD27^+$ $CD28^+$ $CCR7^+$. After activation cells become $CD45RA^-$ RO^+. Whilst expression of CD45RA/RO used to be used to define naïve and antigen experienced cells, it is now clear that cells can revert from being $CD45RO^+$ to $CD45RA^+$ (with late differentiated cells expressing CD45RA (Champagne *et al.*, 2001; Hamann *et al.*, 1997)), although it is not clear what this switch signifies.

CD27 and CD28 are co-stimulatory molecules which provide signals needed for activation of T cells (Hendriks *et al.*, 2000; June *et al.*, 1994; Lenschow *et al.*, 1996). After interaction of CD27 and CD28 with their ligands, T cells no longer require a signal through these molecules and CD27 and CD28 are downregulated (Azuma, 1993; Hintzen, 1993). A gradual loss of CD28 followed by CD27 is thought to occur as cells mature to fully differentiated cells which are $CD28^-$ $CD27^-$ (Hamann *et al.*, 1997).

Two subsets of memory cells can be distinguished on the basis of differential expression of CCR7. CCR7 is a chemokine receptor which is necessary for lymphocytes to cross the high endothelial venule barrier to enter lymph nodes, and therefore has a role in mediating homing of cells to lymphoid sites of infection (Campbell *et al.*, 1998; Forster *et al.*, 1999; Gunn *et al.*, 1998). Naïve cells are $CCR7^+$, whereas antigen-experienced cells can either be $CCR7^+$ or $CCR7^-$ (Sallusto *et al.*, 1999). Memory cells which lack expression of CCR7 are termed effector memory cells; they migrate to peripheral tissues where they have immediate effector function. Memory cells which do express CCR7 are termed central memory cells; they have the potential to home to secondary lymphoid organs where they have little effector function, but possess greater proliferative potential (Sallusto *et al.*, 2004; Sallusto *et al.*, 1999). It is unclear what the lineage relationship between these may be (if any). Two models of linear differentiation of $CD8^+$ T cells have been proposed: Naïve → central memory → effector memory → effector (Champagne *et al.*, 2001; Sallusto *et al.*, 1999) and naïve → effector → effector memory → central memory (Wherry *et al.*, 2003b).

There have been numerous studies that have addressed the phenotype of $CD8^+$ T cells in persistent viral infections. These have indicated that during the primary stage of infection virus-specific $CD8^+$ T cells are predominantly $CD45RA^-/RO^+$; are generally $CCR7^-$, and may have down-regulated expression of CD28 and/or CD27, i.e. are activated, and contain a high proportion of effector or effector/memory cells, which in some cases may be at early stages of differentiation (Appay *et al.*, 2002).

As infections progress into chronicity, greater heterogeneity becomes apparent, with virus-specific T cells retaining an 'immature' phenotype in some cases, but a proportion of cells acquiring a 'later differentiation' phenotype in other infections. For example in primary HCV infection, HCV-specific T cells have a $CD45RA^-$ $CD27^+$ $CCR7^-$ phenotype (Urbani *et al.*, 2002), and remain $CD27^+$ (and $CD28^+$) during chronic infection (Appay *et al.*, 2002).

While the majority of EBV-specific cells in primary infection have been reported to have an activated phenotype ($CD45RO^+$) (Callan *et al.*, 1998a), primary EBV infection has also been reported to also induce the differentiation of $CD27^-$ $CD28^-$ cells (Roos, 2000). Later on, during the persistent phase of infection, the frequency of $CD45RA^+$ cells has been found to increase, with a corresponding decrease in $CD45RO^+$ cells (Callan *et al.*, 1998a). A difference

in the differentiation of T cells (depending on the source of the epitope) was later discovered, with T cells specific for both lytic and latent epitopes during primary EBV infection being $CCR7^-$ $CD45RO^+$, but during persistent infection, those T cells specific for lytic epitopes acquired CCR7 expression, and were $CCR7^+$ $CD45RO^+$, whereas T cells specific for latent epitopes had differentiated into $CCR7^-$ $CD45RA^+$ cells (Hislop *et al.*, 2002).

In primary CMV infection (which is often difficult to study given its asymptomatic nature), cells were found to be predominantly $CD45RO^+$ $CD28^-$ $CCR7^-$. During convalescence, an expansion of $CD45RA^+$ cells was observed, and in the memory pool, virus-specific $CD8^+$ T cells were abundant in both the $CD45RO^+$ and $CD45RA^+$ subsets, and both $CD27^+$ $CD28^-$ and $CD27^-$ $CD28^-$ populations (Wills, 2002).

In studies of chronic EBV and CMV infection, the general consensus is that in healthy (HIV seronegative) subjects the majority of EBV and CMV-specific cells have a memory phenotype ($CD45RO^+$ $CD27^+$) (Appay *et al.*, 2002; Gamadia *et al.*, 2001; Gillespie *et al.*, 2000; Hislop *et al.*, 2001) with low levels of effector phenotype cells (van Baarle *et al.*, 2002b) (although others have found that EBV-specific (Roos, 2000) and CMV specific (Appay *et al.*, 2002) $CD8^+$ T cell populations can include significant numbers of $CD27^-$ cells, and may exhibit other features characteristic of more differentiated cells, e.g. there are reports of $CD45RA^+$ $CD27^-$ $CD28^-$ $CCR7^-$ CMV-specific cells (Sacre *et al.*, 2005; Tussey *et al.*, 2003)).

Analysis of EBV- and CMV-specific T cells in HIV-infected individuals has suggested that a higher proportion of CMV-specific cells in HIV-infected patients have a late differentiation pattern ($CCR7^-$ $CD45RA^+$ $CD27^-$ $CD28^-$) (Appay *et al.*, 2000; Champagne *et al.*, 2001; Chen, 2001; Doisne *et al.*, 2004; Ellefsen *et al.*, 2002; Sacre *et al.*, 2005), with mixed phenotypes reported for EBV-specific cells: $CD45RA^{+/-}$ $CD27^+$ $CCR7^-$ (Chen, 2001; Doisne *et al.*, 2004; van Baarle *et al.*, 2002b) (although some reports suggest that a substantial proportion of EBV-specific cells can be $CD27^-$ (van Baarle *et al.*, 2002b)). The differences in reported phenotype of CMV and EBV-specific cells in healthy donors compared to those co-infected with HIV may reflect differences in recent antigen encounter. The memory phenotype of EBV and CMV-specific cells in healthy donors suggests that they have not recently encountered antigen. In HIV-infected donors, a higher proportion of EBV and CMV cells have downmodulated CD27 and there is a trend towards more expression of CD45RA suggesting that the cells may have been more recently activated in

HIV-infected individuals compared to in healthy donors. This may reflect bystander activation or reactivation of EBV and CMV in the context of HIV-associated immununosuppression.

Multiple studies have indicted that HIV-specific cells in chronically-infected individuals show signs of incomplete differentiation when compared to CMV and EBV-specific cells, with HIV-specific cells having a $CD45RA^-$ $CCR7^-$ $CD27^+$ $CD28^{+/-}$ phenotype (Appay *et al.*, 2000; Appay *et al.*, 2002; Champagne *et al.*, 2001; Chen, 2001; Ellefsen *et al.*, 2002; Ogg, 1999; Scott-Algara *et al.*, 2001; Shankar *et al.*, 2000; van Baarle *et al.*, 2002b). Limited studies of HIV-specific CD8 cells in primary infection have found that a $CD45RA^-$ $CCR7^-$ $CD27^+$ $CD28^-$ phenotype is also apparent at this time (Appay, 2002; Doisne *et al.*, 2004; Papagno *et al.*, 2004).

It has also been suggested that HIV-specific T cells may be functionally immature, in addition to phenotypically immature (Appay *et al.*, 2000; Appay *et al.*, 2002; Champagne *et al.*, 2001). Many studies have reported defects in the cytolytic ability of HIV-specific $CD8^+$ T cells (Appay *et al.*, 2000; Shankar *et al.*, 2000; Trimble & Lieberman, 1998). Furthermore, HIV-specific $CD8^+$ T cells were reported to contain less perforin compared to that of the CMV-specific T cells from the same subject in both primary (Doisne *et al.*, 2004) and chronic HIV infection (Appay *et al.*, 2000) (and this was associated with poor *ex vivo* killing ability compared to that of the CMV-specific cells which did express perforin (Appay *et al.*, 2000)). Other studies are in agreement that HIV-specific cells in the periphery have a low level of or lack perforin expression (Appay, 2002; Appay *et al.*, 2002; Shankar *et al.*, 2000). Furthermore, perforin was found not to be expressed in T cells in lymph nodes during primary and chronic HIV infection (Andersson *et al.*, 1999; Andersson *et al.*, 2002).

It is unclear whether the $perforin^-$ phenotype is a result of accumulation of T cells at a specific stage of differentiation (Champagne *et al.*, 2001), or is reflective of an impairment in T cell responsiveness or functional capacity (anergy) (Lieberman *et al.*, 2002; Zhang *et al.*, 2003a). Another suggestion has been that the absence of perforin is due to the presence of persistent viral antigen triggering degranulation of cytotoxic granules of HIV-specific CTL. However, degranulation would deplete granzymes too, but an abundance of cells that are $granzyme^+$ in the lymph nodes argues against recent degranulation as being an explanation for the observed lack of perforin expression (Andersson *et al.*, 1999). Also, in those patients on therapy, low

levels of perforin are still found despite suppression of the viral load to undetectable levels.

Although, as described above, some studies have addressed the phenotype of HIV-specific T cells in primary infection, only a limited number of patients and epitope-specific responses were studied, and therefore it remains unclear whether the observations made are representative of most patients and epitope-specific responses, and whether there are any differences in patients who subsequently control viral replication with differing efficiency. Here, I used HIV-specific tetramers and antibodies against relevant markers to follow the phenotype of expanded populations of HIV-specific cells from primary infection onwards. This allowed me to see if the pre-terminally differentiated phenotype of cells in chronic infection was also characteristic of most cells present during primary infection (as others previously observed for the limited responses studied (Appay, 2002; Papagno *et al.*, 2004)), or to follow the kinetics with which cells of this phenotype are acquired during infection. Different epitope-specific responses in different patients were studied to address whether cells responding to different epitopes may differ in phenotype and how the phenotype of HIV-specific T cells may relate to the persisting viral load established in different individuals.

In primary infection, most HIV-specific $CD8^+$ T cells were found to be $CD45RA^-$ which is consistent with them having recently been activated. A low percentage of $CD45RA^+$ cells was detected (possibly CD45RA revertants). The tendency (for most epitope-specific responses) was for all cells to be $CD45RA^-$ to start with, and then a low percentage of $CD45RA^+$ cells to appear over time. Interestingly, some of the responses which expanded later seemed to have a proportion of $CD45RA^+$ cells present from early within the response onwards. The reasons for this are unclear. There was no difference in CD45RA expression between different patients/epitopes. The implications of these observations in terms of functional efficacy are not clear.

Results from looking at the expression of CD27 on HIV-specific cells were consistent with studies in chronic infection (Appay *et al.*, 2000; Ogg, 1999; Shankar *et al.*, 2000; van Baarle *et al.*, 2002b): it was found that CD27 was retained on a high proportion of HIV-specific cells of all antigenic specificities in all patients. Absence of $CD27^-$ terminally differentiated cells within the tetramer-stained populations may imply that, despite continuous activation of cells by persistent antigen, there is a defect in the maturation of cells and the

generation of effector cells. Persistent CD27 expression on HIV-specific cells was seen from the earliest timepoint studied onwards, implying that incomplete maturation of cells is a feature of primary infection onwards. In contrast to some reports (Appay *et al.*, 2000; Shankar *et al.*, 2000; van Baarle *et al.*, 2002b), CD28 was found on a proportion of HIV-specific cells. However, there was evidence of cells losing expression of CD28 by the later timepoints during the responses to IPRRIRQGL in MM12 and ERYLKDQQL in SUMA, suggesting that for these two responses the cells may be able to progress to a later stage of maturation at later timepoints. That this was true of two responses which were not initially immunodominant in the two subjects with the lowest persisting viral loads may lead to speculation about an association between the ability of the cells to mature to the $CD28^-$ stage of differentiation, and the persisting viral load of the subject (or the relative dominance of responses). Additional responses would need to be analysed in order to explore this further. A previous report also suggested that T cells responding to different epitopes within the same patients may have differing phenotypes (in this case, differing in terms of the proportion of $CD27^-$ cells) implying that cells specific for one epitope may have differentiated more efficiently into T cells with an effector phenotype with corresponding functional capacity than cells specific for another epitope restricted by the same HLA allele (Kostense *et al.*, 2001). However, the relative dominance of these two responses in the patients studied was not indicated.

In one study which followed the phenotype of HIV-specific cells throughout the course of infection from the primary stage to the chronic stage (Appay, 2002), during primary infection, high numbers of $CD28^+$ $CD27^+$ cells were observed (likely to be generated from a population of naïve cells) which differentiated rapidly (within 2-4 weeks) into an intermediate stage of differentiation, where cells were $CD28^+$ $CD27^-$. The chronic/memory phase showed an enrichment of $CD28^+$ $CD27^-$ cells mixed with smaller numbers of $CD28^+$ $CD27^+$ cells and $CD28^-$ $CD27^-$ cells. The observations made here were similar, although the kinetics of CD28 loss were typically slower than observed by Appay *et al.*

HIV-specific $CD8^+$ T cells in the circulation were found to be universally $CCR7^-$ irrespective of time, specificity or persisting viral load. This could imply that $CCR7^+$ cells are all localised within the lymph nodes (and so cannot be detected when analysing HIV-specific cells using peripheral blood samples), or that there is a lack of the $CCR7^+$ central memory cell subset. In support of the latter explanation is one study in which, looking at the simultaneous

expression of CCR7 and CD45RA on total peripheral blood $CD8^+$ T cells, HIV-infected donors were found to have a marked reduction in $CCR7^+$ naïve and central memory cells within their CD8 lymphocyte population (Chen, 2001). In the same study, a lack of lymph node homing receptors on HIV-specific cells (and also EBV- and CMV- specific cells within the same donor) during chronic infection was also observed, and was found to be associated with a reduction in the frequency of antigen-specific cells in the lymph nodes. Therefore the lack of expression of CCR7 on HIV-specific cells may affect their ability to traffic efficiently and localise at principal sites of HIV replication, and thus interfere with their ability to provide protection. A study of primary SIV infection also suggested that SIV-specific $CD8^+$ T cells were excluded from lymph nodes (Kuroda *et al.*, 1999). That CMV and EBV-specific cells were also deficient in CCR7 expression in HIV-infected patients and healthy donors (Chen, 2001) suggests that in the setting of chronic infection and persisting antigenic stimulation, antigen-experienced CD8 cells may be selectively excluded from lymph nodes. This may serve as a protective mechanism against immunopathological damage caused by inflammatory cytokines and cytolytic enzymes.

That there are cells of an early differentiated phenotype present during HIV infection may not be an adverse characteristic of the response, for such cells may have better proliferative ability and be associated with protective immunity, as described in LCMV infection (Wherry *et al.*, 2003b). Also, in HIV infection it was shown that the presence of HIV-specific cells which maintained a strong proliferative capacity following *in vitro* stimulation (cells defined mainly as $CD45RO^+$ $CD28^+$ $CD27^+$ early differentiated cells) and were able to express high levels of perforin under certain conditions of stimulation was observed in non-progressor subjects (Migueles *et al.*, 2002). This raises questions regarding the ideal phenotypic profile of 'protective effector cells'. Should vaccination strategies be designed to favour expansion of perforin-high, late differentiated cells when these cells could have characteristics of replicative senescence? In line with this, in contrast to thinking of loss of CD28 expression as being associated with a 'good' effector phenotype, it has been suggested that that the accumulation of $CD28^-$ HIV-specific cells during disease progression is not a favourable thing (Gamberg *et al.*, 2004). A predominance (and accumulation) of $CD28^-$ cells reflects continuous differentiation of $CD28^+$ cells in response to chronic viral stimulation, depleting

reserves of potential effector cells within the $CD28^+$ population. This could have a negative impact on the ability to recruit new effector cells from the $CD28^+$ population.

Contrastingly, in support of late differentiated cells being more beneficial, in patients with recent acute infection, the expansion of activated CD8 cells that were $CD57^+$ or $CD45RA^+$ was found to be inversely correlated with a reduced viral setpoint after primary infection (Lieberman *et al.*, 1999), suggesting that the cells displaying properties of later stages of differentiation may be associated with an ability to control the virus. Furthermore, consistent with virus-specific $CD27^-$ cells having stronger effector function compared to $CD27^+$ cells, higher numbers of HIV-specific $CD27^-$ cells have been associated with delayed progression to AIDS (van Baarle *et al.*, 2002b). However, it was suggested that a large number of highly differentiated cells may simply accumulate over time in chronic infection, whilst playing no part in delaying disease progression (Papagno *et al.*, 2004).

In order to find out more about the turnover of HIV-specific cells, the expression of Ki67 and CD57 on tetramer-labelled cells were also followed. Ki67 is an intracellular marker of proliferation, expression of which is restricted to cells in cycle (Gerdes, 1984) and so the proportion of $Ki67^+$ cells can be used as a measure of T cell production and turnover. Ki67 has been found to be expressed on a high proportion of cells in primary HIV infection (Appay, 2002; Doisne *et al.*, 2004; Papagno *et al.*, 2004), and on a lower proportion of cells in chronic HIV infection (Appay *et al.*, 2000; Champagne *et al.*, 2001). As expected, I observed that Ki67 expression in HIV-specific $CD8^+$ T cells was mainly detected during the expansion phase of each response when cells were proliferating, and subsequently decreased.

$CD8^+$ $CD57^+$ cells are thought to represent a terminal differentiation state of activated effector CTL which are enriched in antigen-specific populations and have down-modulated their cytotoxic potential (Mollet *et al.*, 1998). As CD8 cells differentiate further, they express increasing levels of CD57, and so this molecule has been used as a marker of proliferative inability or replicative senescence (Bandres *et al.*, 2000; Weekes *et al.*, 1999). Here, it was found that cells did not express CD57 as the HIV epitope-specific response expanded, but as each response progressed, there was expression on an increased proportion of cells. The increasing proportion of cells expressing CD57 is likely a consequence of continual stimulation by antigen/cytokines,

with $CD57^+$ cells having a long proliferative history and a reduced ability to proliferate further. In line with my observations, a study of HIV-specific cells found them to be $CD57^-$ during primary infection, but showed that CD57 expression increased following primary infection as disease progressed (Papagno *et al.*, 2004), suggesting that T cells exhibit characteristics of replicative senescence as they differentiate further with increasing stage of infection.

Cytokines such as IL-2 and IL-7 are stimulatory cytokines which can affect T cell maturation and differentiation, enhance $CD8^+$ T cell proliferation and cytolytic activity (Carini & Essex, 1994; Carini *et al.*, 1994), and also have anti-apoptotic effects (Estaquier *et al.*, 1996). It has also been shown, using an LCMV infection model, that the expression of the IL-7R α chain distinguishes the subset of cells in the acute $CD8^+$ T cell response that will differentiate into memory cells (Kaech, 2003; Huster 2004).

It could be hypothesised that the interruption of essential cytokine signalling pathways may underlie the impairment of CTL activity in HIV disease. As suggested by other studies (Vingerhoets *et al.*, 1998), the mechanism involved could involve the expression of cytokine receptors since downregulation of receptor expression may result in signalling defects. Indeed, IL-7R expression was found to be reduced in HIV-infected individuals compared to in healthy controls, and improved immune function with effective antiretroviral therapy was associated with recovery of expression of this molecule (MacPherson *et al.*, 2001).

CD25 is the high affinity IL-2Rα chain which is important in regulating the T cell response to antigen (Nelson & Willerford, 1998). It was also found that cells from HIV-infected individuals were unable to upregulate expression of CD25 upon antigenic stimulation (Vingerhoets *et al.*, 1998), so this defective CD25 expression may be involved in T cell unresponsiveness. It has been suggested that absence of a co-stimulatory signal induced by IL-2R engagement may be linked to the deficiencies in perforin expression in HIV-specific $CD8^+$ T cells in infected individuals (Zhang *et al.*, 2003a). The defect in expression of CD25 upon activation may be secondary to defects in key signalling molecules in HIV-specific $CD8^+$ T cells. CD3ζ and CD28 have been found to be down-regulated on HIV-specific $CD8^+$ T cells (Trimble *et al.*, 2000). This may raise the threshold for induction of functions induced by TCR engagement, such as cytotoxicity (the reduced cytotoxicity of freshly isolated

$CD8^+$ T cells from HIV-infected subjects has been shown to be linked to the downmodulation of CD3ζ (Trimble & Lieberman, 1998)). Indeed, upon activation, cells with decreased CD3ζ and CD28 did not express CD25 (Trimble *et al.*, 2000).

In the studies here of different epitope-specific responses in different patients, neither IL-7R nor CD25 expression were detected on a large part of any epitope-specific population. The absent expression of cytokine receptors implies that HIV infection may cause down-modulation of their expression, leading to signalling defects in $CD8^+$ T cells and altered responses to stimulatory cytokines. However, the failure to detect CD25 expression on any CD8 population raises suspicion as to whether the antibody was working properly (although this antibody was found to stain a proportion of HIV-specific cells in earlier experiments (data not shown)). To determine whether the antibody was no longer working or whether CD25 was universally not expressed, the antibody could have been used to stain cells activated *in vitro* by stimulation with PHA.

Functionally defective and phenotypically immature HIV-specific responses could result from problems with the initial priming of the response and/or with maintenance of the response over time once naïve cells have been activated. The fact that an 'immature' phenotype was detected from the initial expansion of the response in primary infection suggests that the problems arise from the priming stage onwards (although there may be problems with maintenance too). Using CD27 and perforin expression to examine the maturation status of HIV-specific cells, Janssen *et al* also concluded that the impaired maturation of HIV-specific cells was established 'early' on in infection (Jansen *et al.*, 2004) (although this study only compared cells at two timepoints: 'early' and 'late', early here being defined as 1-2 years following seroconversion and late around the time of AIDS diagnosis, or in the case of slow progressors, 10 years after seroconversion). Partial differentiation (as reflected by a slight increase in perforin expression in HIV-specific cells over time) was however found to be possible in those individuals with slowly progressing infection.

What factors could be important in influencing the failure of HIV-specific cells to differentiate fully? T cell activation/differentiation are known to be influenced by antigen exposure, by signals received from APCs and $CD4^+$ T cells, and by soluble factors including type I IFNs, IL-15 and IL-2. The failure of $CD8^+$ T cells

to mature and function properly may be a consequence of perturbation of any of these factors.

Antigenic stimulation is a major driving force for T cell activation. During chronic viral infections, the antigen burden is thought to be a key determinant of the activation state of virus-specific $CD8^+$ T cells. Tussey *et al* observed that memory phenotype cells tended to predominate in chronic viral infections where antigen loads were absent or low (such as in EBV infection), and significant numbers of terminally differentiated cells were present when the antigen burden was higher (such as in CMV infection) (Tussey *et al.*, 2003). This led to the construction of a dynamic model of T cell differentiation with virus-specific T cells switching between subsets in response to changes in plasma viral load. It was suggested that when the antigen burden is low, cells mature to memory, but during recrudescence, some of these memory cells are recruited back to the more activated subset of $CD45RO^+$ $CD27^+$ $CD28^-$ cells (i.e. a phenotype associated with acute phase expansions). This was supported by longitudinal analysis of HIV-infected patients, in which a shift towards memory was observed as viral load decreased and reversion back to an acute expansion phenotype as viral load increased (Tussey *et al.*, 2003); although another study found no relationship between levels of HIV plasma viral load and the differentiation phenotype of HIV-specific $CD8^+$ T cells (Appay *et al.*, 2002). This may also explain the differences in the phenotype of EBV-specific cells in healthy carriers and HIV-infected subjects, with EBV-specific cells in the context of HIV infection being triggered to undergo differentiation into a more mature phenotype (van Baarle *et al.*, 2002b).
During acute/early HIV infection, very high antigen loads are present, yet HIV-specific $CD8^+$ T cells do not acquire a 'fully mature' $CD45RO^+$ $CD27^-$ $CD28^-$ phenotype. One explanation for this is that high persistent antigenaemia may result in impairment of CD8 function (Lieberman *et al.*, 2002).

However antigenic stimulation is not the only factor that drives T cell differentiation/maturation. In addition to antigen, APCs and $CD4^+$ T cells have an important role in influencing $CD8^+$ T cell differentiation.
As reviewed earlier, numbers of both conventional and plasmacytoid DCs in the blood are reduced in primary HIV infection (Kamga *et al.*, 2005; Pacanowski *et al.*, 2001; Soumelis *et al.*, 2002), and there is evidence that there may also be impairments in the function of PDCs and conventional DCs

(Donaghy *et al.*, 2003; Feldman *et al.*, 2001; Kamga *et al.*, 2005; Lore *et al.*, 2002). In addition, monocytes/macrophages may have reduced expression of molecules required for effective stimulation of T cells (e.g. MHC class II and CD80/CD86 (Clerici *et al.*, 1991)), and increased expression of molecules that can trigger apoptosis such as Fas and FasL (Badley *et al.*, 1996). Defects in innate cytokine production by DCs and abnormalities in contact-dependent interactions between T cells and DCs could result in incomplete or aberrant T cell stimulation, leading to the observed failure of HIV-specific $CD8^+$ T cells to acquire a fully mature phenotype in primary HIV-1 infection.

$CD4^+$ T helper cells also provide help for expansion and maintenance of fully functional $CD8^+$ T cell responses through their ability to provide co-stimulatory and cytokine support. In support of this, cells responding to LCMV infection in mice which lacked helper T cells, were found to be deficient in function (Zajac *et al.*, 1998). In HIV-infection the enhancement of HIV-specific $CD8^+$ T cell function *in vitro* after short term incubation with IL-2 (Shankar *et al.*, 2000; Trimble & Lieberman, 1998; Trimble *et al.*, 2000) also supports that a lack of CD4 help may contribute to the functional impairment of HIV-specific $CD8^+$ T cells. Associations between the degree of functional defect and disease stage/CD4 count (Shankar *et al.*, 2000), and the presence of preserved virus-specific CD4 function in those with $CD8^+$ T cells with fully effector/a more differentiated phenotype (Hess *et al.*, 2004; van Baarle *et al.*, 2002b), suggests that maturation of virus-specific $CD8^+$ T cells may be linked to preservation of HIV-specific $CD4^+$ T cell function.

Since HIV-specific $CD4^+$ T cells are preferentially infected by HIV (Douek *et al.*, 2002) and the initial loss of help is specific for HIV antigens during primary HIV infection (Musey *et al.*, 1999), this may explain why HIV-specific cells fail to acquire a fully differentiated phenotype from primary infection. By contrast, when the response to e.g. CMV is primed, adequate $CD4^+$ T cell help is likely present. The eventual loss of functional CMV-specific CD8 cells associated with end stage AIDS disease when CD4 counts are severely diminished reflects a universal loss of CD4 helper function. This, together with the observation that in patients with very low CD4 T cell counts, even CMV-specific cells had no direct virus-specific effector activity (Spiegel *et al.*, 2000), further support that CD4 help is an important contributing factor to $CD8^+$ T cell maintenance.

However, the observations that even in those exhibiting high HIV-specific CD4 responses in primary infection, HIV-specific $CD8^+$ T cells did not differentiate

further (Appay, 2002), and that $CD27^+$ $CD28^-$ cells were found even in LTNPs or those with therapy-induced viral suppression (both situations where help may be preserved) (Appay *et al.*, 2002), do not support the idea that CD4 help is the major determinant of CD8 cell differentiation.

Another proposed explanation for the apparent defect in maturation or functionality of HIV-specific cells is that chronic antigen exposure may cause the immune system to go into a protective mode to protect against potential damage caused by such chronic activation. In accordance with this, some of the reported defects have been found to be apply to T cells in other chronic virus infections, and not just for HIV. Perforin expression was found to be low/absent in the same HIV-infected donor in most cells specific for HIV, CMV or EBV stained *ex vivo* without restimulation (Appay *et al.*, 2002; Sandberg *et al.*, 2001; Zhang *et al.*, 2003a). The associated impaired cytotoxicity of cells that had repeatedly seen antigen (Zhang *et al.*, 2003a) was thought to protect against damage that could arise by T cell recognition and destruction of uninfected cells displaying weakly reactive self peptides. The universal lack of CCR7 expression on HIV, CMV and EBV-specific cells (Chen, 2001) also supports for a mechanism for protecting against immunopathology.

The phenotype of virus-specific CD8 cells thus varies in different virus infections, and it is not clear what exactly is optimal. Although it may be that viruses block/divert T cell differentiation as part of their evasion of the host immune response, it has also been proposed that distinct patterns of differentiation may result from different routes of antigen exposure or particular homeostatic environments occurring in different virus infections, and that the phenotype of the cells primed may represent an appropriate response to the virus. In primary HIV infection, I found that the majority of virus-specific $CD8^+$ T cells had a $CD45RA^-$, $CCR7^-$, $CD27^+$, $CD28^{+/-}$ phenotype similar to that described in chronic HIV infection, which has been proposed to constitute an incompletely differentiated phenotype. However even in the case of EBV and influenza virus infection, viral infections which are controlled, virus-specific $CD8^+$ T cells can be found at an early stage of differentiation (Appay *et al.*, 2002). The precise phenotype of an ideal protective virus-specific response, and whether the phenotype of HIV-specific $CD8^+$ T cells is indeed reflective of a suboptimal protective capacity therefore remain unclear.

In summary, in this chapter, epitope-specific components of the HIV-specific $CD8^+$ T cell response were studied using fluorescently labelled multimeric peptide-MHC complexes to build upon initial observations made in chapter 3 about the nature of the response expanded in different patients. This revealed that $CD8^+$ T cell responses of very high magnitude can be expanded in primary HIV infection in patients who establish both high and low persisting viral loads. Analysis of the kinetics of expansion of epitope-specific $CD8^+$ T cell responses in primary HIV infection showed that in many patients there is a period of ~ 5-6 weeks from the peak in acute HIV replication until the time when the earliest HIV-specific $CD8^+$ T cell response (that was studied here) was found to reach its peak. Furthermore, in the majority of patients studied, different epitope-specific components of the response expanded with differing kinetics, such that an overall picture of asynchronous expansion of different responses emerged. Interestingly, the pattern of expansion of responses was somewhat different in the one patient studied who established a low persisting viral load, in whom a rapid, more synchronous expansion of responses was observed. Other similar patients would need to be studied to determine whether this is a feature of the response which differentiates all patients who are able to control primary viral replication efficiently.

Analysis of the changes in the clonal composition of epitope-specific T cell responses by TCR Vβ family analysis revealed there to be a heterogeneous pattern of evolution of Vβ family usage over time. When increasingly preferential usage of one particular family was observed over time, this was found to be coincident with an increase in the avidity of the response.

Phenotypic analysis of tetramer/pentamer-labelled cells revealed, in all patients and for both dominant and subdominant responses, there to be a lack of $CD8^+$ T cells possessing what is thought to constitute a fully differentiated phenotype. HIV-specific cells were found to retain expression of CD27 (and CD28) from timepoints corresponding to acute infection onwards, although the functional significance of this observation remains to be determined. Overall, these results suggest that factors other than just the magnitude of the primary HIV-specific $CD8^+$ T cell response, potentially including kinetic and/or qualitative aspects of the response, may have a key impact on the efficiency of control of acute/early virus replication.

Chapter 6
General discussion

The events which take place in the early stages of HIV-1 infection are critical determinants of the subsequent disease course: a setpoint level of persisting virus is established at this time that is predictive of the rate of progression to AIDS (Mellors *et al.*, 1996). Although the immune response mounted following infection fails to mediate virus clearance, there is good evidence to suggest that antiviral immunity, in particular the HIV-specific $CD8^+$ T cell response, does play an important role in containment of early viral replication.

This includes the loss of control of virus replication following $CD8^+$ T cell depletion of SIV-infected macaques (Jin *et al.*, 1999; Schmitz *et al.*, 1999); the selection of viral variants able to escape $CD8^+$ T cell recognition in both HIV-infected individuals (Borrow *et al.*, 1997; Goulder *et al.*, 1997a; Jones *et al.*, 2004; Price *et al.*, 1997) and SIV-infected macaques (Allen *et al.*, 2000b; Evans *et al.*, 1999); and the association of certain HLA class I alleles with the rate of disease progression (Carrington *et al.*, 1999; Kaslow *et al.*, 1996).

Consequently, the enhancement/induction of HIV-specific $CD8^+$ T cell responses is the goal of many prophylactic and therapeutic strategies currently in development (review in (Garber *et al.*, 2004)). However, our current picture of the early HIV-specific CD8 response is very incomplete, and it is still unknown what kind of response is able to provide the best long term control of viral replication. Such knowledge would help inform the design of more effective T cell-based vaccines. In consideration of this, the aim of this project was to characterise the HIV-specific $CD8^+$ T cell response in primary infection in a number of HIV-infected individuals to increase our understanding of the response with respect to its magnitude, kinetics, breadth, epitope specificity, and the avidity and phenotype of different epitope-specific T cells. Furthermore, efforts were made to compare these features of the response in patients who naturally controlled early viral replication with differing efficiency in order to identify features of the response associated with poor and good viral control.

More prior studies of the HIV-specific $CD8^+$ T cell response have focused on responses during chronic infection, and not during primary infection. At the time this project was started, there were thus many gaps in our knowledge about the primary HIV-specific $CD8^+$ T cell response. It was known that HIV-specific $CD8^+$ T cell responses can be detected very early in infection, prior to

the peak in acute viral replication (Borrow *et al.*, 1994; Koup *et al.*, 1994); and that individual epitope-specific responses can potentially reach very high magnitudes in primary infection (Borrow *et al.*, 1997; Pantaleo *et al.*, 1994). However the total magnitude of the HIV-specific $CD8^+$ T cell response expanded in primary infection and the extent to which this may vary between patients were not clear. Further, the kinetics of expansion/contraction of the overall response and of epitope-specific components of the response were not well defined. Limited studies had addressed the dynamics of responses to individual viral epitopes in a small number of patients (some of whom were treated with antiretroviral therapy during primary infection) using tetramer staining (Appay, 2002; Wilson *et al.*, 2000), but the immunodominance of the responses followed in these patients was not known, leaving it uncertain as to how representative their magnitude/kinetics were of the dynamics of all epitope-specific components of the primary $CD8^+$ T cell response. Likewise, although some phenotypic analysis of $CD8^+$ T cells involved in the primary response had been carried out (Appay, 2002; Appay *et al.*, 2002; Doisne *et al.*, 2004; Papagno *et al.*, 2004), it was again unclear how representative the findings were of all cells participating in the response. Lieberman *et al* (Lierberman *et al.*, 2001) highlighted the fact that the epitopes used in tetramers are rarely immunodominant (Altfeld *et al.*, 2001b; Betts *et al.*, 2000; Lieberman *et al.*, 1997; Lieberman *et al.*, 1992), and that CD8 cells targeting subdominant epitopes may have lower avidity interactions with target cells than T cells targeting immunodominant responses (Sercarz *et al.*, 1993). Since low avidity interactions may alter T cell activation and differentiation (Leitenberg & Bottomly, 1999), the nature of the epitope studied may influence the conclusions drawn, and so observations should ideally be confirmed using a broad array of tetramers to follow responses to different viral epitopes.

Another aspect of the primary HIV-specific $CD8^+$ T cell response about which limited information was available was its breadth and whether/how this may vary in patients who controlled viral replication with differing efficiencies. Pantaleo *et al* (Pantaleo *et al.*, 1994; Pantaleo *et al.*, 1997b) reported an association between expansion of T cells using TCRs in one or a small number of Vβ families during primary HIV-1 infection and poor control of virus replication. This could reflect differences in the clonality and/or breadth of the primary HIV-specific $CD8^+$ T cell responses in patients who control viral replication with differing efficiency; and/or may reflect differences in the timing with which expansion of epitope-specific responses and/or different clones of

T cells involved in a given response occurs. A small number of studies had addressed the epitope breadth of the primary HIV-specific $CD8^+$ T cell response, although these were typically performed in very early (rather than *acute*) infection (Addo *et al.*, 2003; Cao *et al.*, 2003; Dalod *et al.*, 1999b). These studies indicated that the epitope breadth of the HIV-specific $CD8^+$ T cell response tends to be narrower in primary infection than in chronic infection, but did not observe a relationship between the breadth of the response and concurrent viral load. However they did not address the relationship between the epitope breadth of the $CD8^+$ T cell response in acute/subacute infection and the persisting viral load established by ~six months post-infection.

The approach taken to this study was to initially map the epitope-specificity of the entire HIV-specific $CD8^+$ T cell response in early infection in a panel of infected individuals, and determine the relative magnitude of responses to different viral epitopes at this time (chapter 3); then to use tetramer staining to assess the magnitude and kinetics of selected epitope-specific responses within each patient, the relative dominance of which in early infection was known, and also to study the phenotype of the cells involved in these responses (chapter 5). Efforts were made to perform this analysis in patients who established a range of different persisting viral loads; but unfortunately I had only very limited access to samples from patients who controlled virus replication well. There were also constraints on the number of epitope-specific responses that could be studied in chapter 5. Some of the conclusions made here are thus limited by what could be feasibly investigated. One of the observations made in this study was that the primary HIV-specific $CD8^+$ T cell response in many infected individuals is heavily biased towards a small number of immunodominant epitopes, responses to which may expand more rapidly in acute/subacute infection than responses to subdominant epitopes. Mechanisms that may account for this were explored in chapter 4. The results from each part of this study have already been discussed in detail at the end of the relevant chapter, so here only the main findings are briefly reviewed and discussed; and the implications of these observations for vaccine development are considered.

Tetramer analysis of the dynamics of individual epitope-specific responses in primary HIV infection (chapter 5) revealed that very high magnitude HIV-

specific responses can be induced in primary infection, with 22% of $CD8^+$ cells being found to be directed against a single epitope in one of the patients studied. It was difficult to assess the total magnitude of the entire acute-phase HIV-specific $CD8^+$ T cell response from the tetramer analysis of selected epitope-specific T cell responses carried out; nonetheless, results from both this and other studies (Borrow *et al.*, 1997; Pantaleo *et al.*, 1994) suggest that very high magnitude $CD8^+$ T cell responses can be expanded in primary HIV infection in patients who control viral replication well and poorly. In addition, results from the initial analysis of the magnitude of the 'entire' HIV-specific $CD8^+$ T cell response during early infection (determined by assessing responses to overlapping peptides corresponding to the clade B consensus sequence in IFN-γ ELISPOT assays (chapter 3)), suggested that the magnitude of the total response at this time in different individuals was not significantly related to the level of persisting virus subsequently established, with a high magnitude response being observed in all patients. Likewise other studies have also found no relationship between the magnitude of the HIV-specific CD8 response in primary infection and the viral load (Addo *et al.*, 2003; Cao *et al.*, 2003). This suggests that the magnitude of the response is not the most important determinant of the efficiency of control of viral replication. A study carried out in the LCMV infection model which addressed the magnitude and protective capacity of different epitope-specific CTL responses also found that the magnitude of a response does not necessarily correlate with its protective capacity (Gallimore *et al.*, 1998). Kinetic and/or qualitative aspects of the primary HIV-specific $CD8^+$ T cell response may thus be of greater importance in determining the efficiency with which viraemia is controlled.

When the kinetics of expansion of different epitope-specific responses were compared in different patients (chapter 5), it was found that the first response (of those studied) within each patient was typically relatively slow to develop; the average time between the peak viral load and 'first' epitope-specific response reaching its recorded maximum was 39 days. A delay in the expansion of the HIV-specific CD8 response was also observed in rVV-stimulated IFN-γ ELISPOT assays (chapter 3), but here it was not known whether the very low magnitude responses detected at the earliest timepoints tested was due to the death of highly activated cells in the assay or reflected the presence of a low magnitude response *in vivo* at these timepoints.

However the tetramer studies confirmed that the apparent delay in the response for patient MM27 in the rVV-stimulated IFN-γ ELISPOT assay was a real delay.

Interestingly, the response which showed the fastest kinetics of expansion was observed in SUMA, the patient who established the lowest viral load. This response peaked a week (at the most) after the highest recorded viral load. Considering the kinetics of viral amplification, small changes in the speed of activation and expansion of virus-specific $CD8^+$ T cell responses could have profound effects on viral control (Ehl *et al.*, 1997), so the rapid mobilisation of a virus-specific $CD8^+$ T cell response in patient SUMA (which was also of high magnitude and potentially of high efficacy since it targeted an epitope within the early expressed Tat protein) may be one of the factors that contributed to the ability of this patient to control early viral replication very efficiently. Conversely a delay in expansion of the virus-specific CD8 response may contribute to the ineffective control of early viral replication in the majority of HIV-infected individuals. This has also been suggested to be of importance in HCV infection (Bertoletti & Ferrari, 2003). From studies of the kinetics of virus-specific immune responses in primary HBV and HCV infection, the interval of time between infection and activation of the response was hypothesised to influence the disease profile. In both infections virus-specific $CD8^+$ (and $CD4^+$) T cell responses appeared only after 7-10 weeks after infection (Thimme *et al.*, 2001; Webster *et al.*, 2000). However, the two viruses replicate at different rates, and the slower replication kinetics of HBV allowed sufficient time for adaptive responses to develop, while in HCV infection, viral replication outpaced the development of the adaptive immune response. These differences may contribute to differences in the rate of chronicity for the two infections and explain why HBV is more easily controlled by vaccination.

Another observation made in chapter 5 was that in most (if not all) patients, responses to different viral epitopes expanded asynchronously in primary infection, with more rapidly-expanding responses tending to dominate the initial response. The observation made by Pantaleo *et al* (Pantaleo *et al.*, 1994; Pantaleo *et al.*, 1997b) of monoclonal/oligoclonal TCR Vβ family expansions in patients who controlled viral replication poorly may thus be explained (at least in part) by initial expansion of responses to a limited number of highly immunodominant epitopes. The finding that in patient SUMA (who controlled early viral replication well) the immunodominant earliest

response peaked only one week or less prior to a more subdominant response suggests that responses in this patient may have not only evolved faster but also less asynchronously than in other patients (providing a partial explanation as to why major Vβ family perturbations were not apparent in primary infection in this patient, or other individuals studied by Pantaleo *et al* who controlled early viral replication efficiently).

Results obtained in chapter 3 indicated that during early HIV infection, the HIV-specific $CD8^+$ T cell response in most patients was heavily biased towards a few immunodominant proteins/epitopes. Analysis of the epitope breadth of the response revealed that there was no significant difference in the number of epitopic regions targeted in patients who established persisting viral loads in the three uppermost of the four viral load quartiles (low-intermediate, intermediate-high and high) as defined by Mellors *et al* (Mellors *et al.*, 1996). Unfortunately, no patients who established low persisting viral loads were available for inclusion in this analysis. Previous analysis of the epitope breath of the response in a patient who established a very low persisting viral load (SUMA), showed that in this patient a response of considerable breadth (targeting > 40 epitopes) was induced in primary infection (Jones *et al.*, 2004); however epitope mapping here was carried out using reagents based on the patient's autologous virus sequence, so the results are not directly comparable to those obtained in this study. Whether a broader response is induced in patients who control viral replication most efficiently thus remains unclear. Other studies that have addressed the epitope breadth of the $CD8^+$ T cell response in primary HIV infection have found no association between the number of $CD8^+$ T cell epitopes recognised and the concurrent viral load (Addo *et al.*, 2003; Cao *et al.*, 2003; Dalod *et al.*, 1999b); although these analyses have not addressed the relationship between primary response breadth and the subsequent efficiency of control of virus replication. Notably, a recent study carried out by Addo *et al* in chronically-infected patients found that the breadth and magnitude of responses was no different in LTNPs compared to progessors (Addo *et al.*, 2004).

In summary, available data suggest that the primary HIV-specific $CD8^+$ T cell response in those patients who control virus replication most efficiently may differ from the response made in the majority of infected individuals not in terms of magnitude, but in terms of the kinetics with which it is expanded, with the most rapidly-expanded epitope-specific responses reaching peak

magnitude within a few days of the peak in acute viral replication, and responses of other specificities beginning to expand concurrently and peaking shortly thereafter. By contrast, in many infected individuals there may be a delay of as much as several weeks before the first epitope-specific responses to be expanded reach their peak, and responses of different epitope specificities may expand very asynchronously so that a small number of epitope-specific responses are highly immunodominant during primary infection (although by early infection, the epitope breadth of the response may not be significantly different from that in patients who control virus replication well). The limited analyses of the clonal breadth of epitope-specific T cell responses during primary infection carried out in chapter 3 did not suggest that this differed significantly in patients who controlled virus replication with differing efficiency, although more extensive studies would be required to enable more definitive conclusions to be drawn about this.

Likewise, studies carried out in chapter 5 revealed no differenced in the phenotypic maturation/differentiation state of $CD8^+$ T cells responding to dominant viral epitopes in primary infection in patients who controlled virus replication with differing efficiency. However it is possible that other features of the response that were not analysed here may differ in different infected individuals, such as the proliferative capacity or effector function (e.g. cytolytic capacity or cytokine production) of HIV-specific CD8 T cells. $CD8^+$ T cell proliferative capacity and cytolytic effector functions were found to be preserved in LTNPs (Migueles *et al.*, 2002), indicating that these may be important correlates of good viral control.

Differences in the nature of the primary HIV-specific $CD8^+$ T cell response in patients who establish different persistent viral loads may be a consequence and/or a cause of differences in the level of viral replication.

As discussed in chapters 4 and 5, factors that influence the induction and maintenance of $CD8^+$ T cell responses include antigenic stimulation, interaction with APCs, exposure to innate cytokines/chemokines and $CD4^+$ T cell help.

In primary HIV infection APC numbers may be reduced, and their functional capacity impaired (Lore *et al.*, 2002; Loré & Larsson, 2003; Pacanowski *et al.*, 2001). DCs from HIV-infected patients have been found to have impaired ability to stimulate T cell proliferation (Donaghy *et al.*, 2003; Knight & Patterson, 1997). *In vitro* experiments using a HIV-infected human DC line

showed that the inability to stimulate allogeneic T cell proliferative responses may be due to multiple factors including IL-10 and gp120-induced apoptosis (Beuria *et al.*, 2005).

There are also abnormalities in the number and functional capacity of PDCs during primary HIV infection (Kamga *et al.*, 2005; Pacanowski *et al.*, 2001). These cells act as an important source of type I IFNs and potentially also other innate cytokines involved in $CD8^+$ T cell activation and homeostasis.

HIV-specific $CD4^+$ T cell responses are impaired from primary infection onwards (Gloster *et al.*, 2004; Musey *et al.*, 1999; Oxenius *et al.*, 2000), so there is likely to be a paucity of help for $CD8^+$ T cell activation.

In the context of high level primary viral replication, these defects in immune functions will likely be most severe and $CD8^+$ T cell activation/expansion most markedly compromised. In addition, a high antigen load in itself may lead to dysfunction of CD8 cells due to sustained TCR signalling in the absence of appropriate co-stimulation. As discussed in chapter 4, there may be preferential expansion of selected $CD8^+$ T cell responses (e.g. high affinity responses and/or responses involving cells in the memory pool), with associated suppression of other responses. Potential reasons to explain why certain responses are able to preferentially expand and come to be more dominant early on in infection were explored in chapter 4. One such reason was that certain responses may be less dependent on help for their expansion (Franco *et al.*, 2000), and so under conditions where CD4 help is limiting, they preferentially expand. It was suggested that help-independent responses may be impaired in their ability to differentiate into memory cells (Kan-Mitchell *et al.*, 2004), and so this is another reason as to why the biasing of the response to a limited number of epitopes may be detrimental. Another reason is that the dominant response may suppress the expansion of other responses (Rodriguez *et al.*, 2002; Sette & Fikes, 2003). In line with this, in another study of HIV infection, it was suggested that CD4 cells were needed for effective *in vivo* activation of CTL and that lack of help prevents a broad response from evolving (Hay *et al.*, 1999).

By contrast, in patients where viral replication is initially lower (perhaps due to properties of the infecting virus isolate, host genetics and/or features of the innate response), defects in APC functions and the HIV-specific $CD4^+$ T cell response will be less severe. For example, strong HIV-specific $CD4^+$ T cell responses have been observed in primary infection in patients such as SUMA who control early virus replication well (Gloster *et al.*, 2004), and are also

preserved in LTNPs (Rosenberg *et al.*, 1997). In addition, the number of peripheral blood PDCs and MDCs in LTNPs were found to be unaltered compared to those in control individuals, whereas in progressors numbers were severely decreased (Almeida *et al.*, 2005). Under conditions where APC function and help are relatively preserved, synchronous expansion of $CD8^+$ T cells of multiple specificities may be supported. Interestingly, HIV-specific cells in patient SUMA who had preserved CD4 function, were not more terminally differentiated than those in other patients, suggesting even where CD4 help is present, incomplete differentiation of HIV-specific $CD8^+$ T cells still occurs.
Supporting this hypothesis, it is notable that rapid, synchronous expansion of virus-specific $CD8^+$ T cells of multiple epitope specificities is observed in acute LCMV infection of mice, where APCs are rapidly activated to support CD8 T cell priming (Montoya *et al.*, 2005; Murali-Krishna *et al.*, 1998), whereas in HCV-infected individuals where DC functions are thought to be impaired (Anthony *et al.*, 2004; Bain *et al.*, 2001; Sarobe *et al.*, 2002) and CD4 T cell responses are weak (Chang *et al.*, 2001; Gerlach *et al.*, 1999; Thimme *et al.*, 2001; Wedemeyer *et al.*, 2002), virus-specific $CD8^+$ T cell responses expand slowly and asynchronously (Lechner *et al.*, 2000a; Timm *et al.*, 2004), and virus-specific CD8 T cells exhibit an 'immature' phenotype similar to that of HIV-specific CD8 T cells (Appay *et al.*, 2002; Urbani *et al.*, 2002).

The speed with which a high magnitude virus-specific $CD8^+$ T cell response is mobilised is likely to play a critical role in determining the efficiency with which viral replication is contained, as demonstrated by a study by Ehl *et al* (Ehl *et al.*, 1997). When different spleen cell populations (from naïve, acutely-infected and memory mice) were adoptively transferred into recipient mice pre-infected with LCMV, it was found that the CTL from memory mice provided less protection than CTL from acutely infected mice. This difference in the protective efficacy of the transferred cells was likely to have been due to the reactivation time required by the memory cells and the contrasting ability of cells from the acutely-infected donors to provide immediate effector function. Thus in this situation, a delay in the response had an important impact on the ability of the cells to provide antiviral protection.
Notably however, a subsequent study in which the protective capacity of different subsets of memory T cells was compared in a slightly different adoptive transfer model, where the recipient mice were infected with LCMV following cell transfer, revealed that central memory cells were more effective

than effector memory cells, because of their potential to expand and generate large numbers of effector cells (Wherry *et al.*, 2003b). However whether the time needed for such cells to be activated upon encounter with a virus for which the response kinetics are now thought to be critical, may limit their usefulness remains to be seen.

The breadth (both epitope breadth and clonal breadth) of the $CD8^+$ T cell response induced in primary HIV infection may also have an important impact on the efficiency of control of early viral replication. Viral evolution to escape from epitope-specific CD8 T cell responses is thought to constitute an important immune evasion strategy in HIV-1 infection (Goulder & Watkins, 2004). CTL escape viral variants can start to emerge during the acute phase of infection, and may be selected for to completion in the *in vivo* viral quasispecies within as little as a few weeks (Allen *et al.*, 2000b; Borrow *et al.*, 1997; Jones *et al.*, 2004; O'Connor *et al.*, 2002) – the kinetics and extent of viral escape from the primary $CD8^+$ T cell response may thus potentially have an important impact on the efficiency of control of early viral replication.

CTL escape is promoted when a response makes a particularly important contribution to control of viral replication, due to its magnitude and/or efficacy and immunodominance. It follows that a response which exerts broad and even pressure on the virus will be associated with the least amount of escape, as the benefit to be gained from escape from any one particular component of the response will be less. This was illustrated in a study of the emergence of CTL escape variants in patient SUMA, and two other patients who had less broad, and more biased responses (Jones *et al.*, 2004). There was much less extensive CTL escape in patient SUMA compared to other patients (who established higher persisting viral loads).

Having a response of greater clonal breadth can also be advantageous in limiting viral escape, as suggested by the broad usage of TCR Vβ families by virus-specific $CD8^+$ T cells and their associated cross-recognition of variant epitopes observed in HIV-2-infected patients (Lopes *et al.*, 2003). Such patents typically show a much less severe disease course than HIV-1-infected patients. By providing the potential for recognition of multiple variants of an epitope peptide, greater heterogeneity of TCR usage in an epitope-specific CD8 response may thus help to restrict viral escape.

Therefore, while it could be envisaged that a response which can be rapidly activated could be advantageous in early containment of viral replication, there

is a risk of it becoming significantly immunodominant, to the detriment of the ensuing efficiency of control of virus replication. The rapid kinetics of the initial response of patient SUMA may have contributed to the efficiency of viral control in this patient, but the rapid expansion of HIV-specific $CD8^+$ T cells of other specificities may have prevented the initial response coming to dominate the entire response, and restricted the extent to which the overall response was escaped. Viral escape from CTL recognition may pose a huge threat to the success of to CTL-based vaccines. The emergence of viral mutations that escaped recognition by dominant epitope-specific CTL in vaccinated macaques who initially controlled SIV challenge effectively has been reported (Barouch *et al.*, 2002); such escape was associated with rapid progression to AIDS.

This suggests that to achieve optimal control, a therapeutic or prophylactic vaccine should aim to preserve or elicit CTL responses of evenly high magnitude and efficacy to a series of epitopes (since this will minimise the emergence of CTL escape variants), and that are able to be activated rapidly. This should target invariant, functionally constrained regions in the virus, where escape is likely to be restricted by costs to intrinsic fitness (Kelleher *et al.*, 2001; Wagner *et al.*, 1999), and/or proteins which are expressed early in the viral lifecycle (since these may have a kinetic advantage in recognising HIV-infected cells before new viral progeny have been released (Gruters *et al.*, 2002; van Baalen *et al.*, 2002)).

There is a risk however of skewing the immune response towards immunodominant epitopes when trying to induce an immune response to multiple epitopes simultaneously in prime-boost protocols due to competition of CTL for APC (Palmowski *et al.*, 2002; Rodriguez *et al.*, 2002). In order to induce a broad response, it may be necessary to use separate constructs encoding individual epitopes or proteins for the boosting phase, such that APC separately present the epitopes (Palmowski *et al.*, 2002).

Given the importance of a functional CD4 response any vaccine-elicited CD8 response should be raised in the context of good CD4 function, for insufficient help may impact on the priming and formation of functional memory responses. The virus-specific CD4 response may also act together with other arms of the immune system, such as the antibody response, to contain ongoing viral replication at levels which do not exhaust the CD8 response (Planz *et al.*, 1997). It is also important to preserve APC function to provide appropriate co-stimulation to enable optimal priming of CD8 cells, since a lack

of fully functional APCs could result in the aberrant activation of HIV-specific $CD8^+$ T cells. The co-stimulatory signals provided by DCs are also thought to be important in inducing high avidity CD8 cells (Oh *et al.*, 2003), which are more effective in mediating protective immunity (Alexander-Miller *et al.*, 1996; Gallimore *et al.*, 1998).

That a vaccine-induced virus $CD8^+$ T cell response alone cannot provide long term control of HIV-1 replication, and that the induction of other components of the immune response is important was illustrated in macaques who were immunised using a prime/boost regimen which induced a multi-specific mucosal CTL response but not an Env-specific antibody response, and then subsequently challenged with pathogenic SIV (Vogel *et al.*, 2003); SIV-specific cellular responses were able to control primary viral replication, but without neutralising antibody, the virus-specific response was unable to control chronic phase viral replication.

Overall, the most effective control of HIV replication is thus likely to require the induction of a fully integrated innate, humoral and cell-mediated immune response from the early stages of infection, and this should be the goal of vaccination strategies.

In summary, the results from this thesis suggest the importance of not only the magnitude, but also kinetic and qualitative features of the virus-specific $CD8^+$ T cell response in primary HIV infection in determining the efficiency of control of early virus replication. These findings have a number of implications for both the prophylaxis and treatment of HIV infection.

First, they suggest that among the aims of a successful prophylactic HIV vaccine should be the generation of a pool of central memory cells of multiple epitope specificities that would be present at the time of infection to act rapidly to limit initial viral replication (particularly in the gut mucosal tissues where much of the initial infection and damage of memory CD4 cells takes place (Li *et al.*, 2005; Mattapallil *et al.*, 2005)), and subsequent dissemination around the body. A reduced initial level of viral replication and damage to the CD4 cell compartment may in turn enable increased levels of functional effector CD8 cells to develop with enhanced ability to control viral replication during chronic infection. In addition, prophylactic vaccines should ideally also elicit other responses, including neutralising antibody responses that may contribute to control of virus replication both in the critical early stages of infection and at

later timepoints; and virus-specific $CD4^+$ T cell responses that may be of importance for the induction and maintenance of effective virus-specific $CD8^+$ T cell responses.

Second, they emphasise the importance of events in primary HIV infection in determining the overall disease course, suggesting the value of exploring the potential utility of rapid diagnosis of HIV infection and "acute phase" therapeutic intervention as a means of inducing better long-term control of virus replication.

Third, it is clear that there are shortcomings of the immune response in patients chronically-infected with HIV. Deficits in APC function and/or $CD4^+$ T cell responses, escape from initially dominant/efficacious $CD8^+$ T cell responses and/or defects in $CD8^+$ T cell function may all have occurred by this time, necessitating design of therapeutic strategies to reverse/overcome these defects. The latter presents many obstacles, but is given impetus by findings such as those described here which indicate that at least in a minority of patients, immune responses are induced that mediate sustained control of viral replication over time.

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